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## THE USE OF AN IRON STAIN FOR THE STUDY OF ALVEOLAR DEVELOPMENT IN THE MOUSE MAMMARY GLAND<sup>1</sup>

BY H. E. RAWLINSON

### Abstract

A method is described of using whole mounts of the mouse mammary gland with Gömöri's potassium ferrocyanide - hydrochloric acid mixture for staining iron. Because iron accumulates in the epithelial cells in a granular form, most of the mammary gland tree of the mature nonlactating female mouse can be shown up clearly. The staining reaction is quick and uniform, muscle can be clearly differentiated in microdissection, and alveolar development can be estimated from the amount of iron retention in the nonlactating gland.

### Introduction

The accumulation of histologically visible iron in the glandular cells of the nonlactating mammary gland of mature female mice has been pointed out (4, 5). This development is so marked that the well known technique of staining whole mounts can be used, with the glands being stained en masse for iron and cleared without counterstain. This gives a clear picture of most of the gland tree, particularly its peripheral alveolar part. The advantages of the method are: (1) the speed and uniformity in the action of the staining mixture; (2) the ease with which muscle and other extraneous tissues can be dissected away; (3) the sharpness of the picture that results from the color differentiation and the high degree of clearing in the nonepithelial tissues; (4) the facility with which alveolar development can be assessed because of the parallel that exists between it and iron retention.

### Materials and Methods

In this study female mice of the dba, C57 Black, and C3H strains were used. They were killed, usually by decapitation, and the hides were removed with the mammary glands on them and fixed in 10% neutral buffered formalin (Lillie, 3) for 18 to 24 hr. The gland pads were then dissected free from the skin using glass seekers made from stirring rods, since it is necessary to avoid iron contamination as far as possible until after staining has been accomplished. The pieces of tissue were then soaked in distilled water for an hour or so to

<sup>1</sup> Manuscript received August 26, 1949.

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the University of Alberta.

remove the fixing fluid and dropped into a few cc. of a fresh mixture of 20% hydrochloric acid and 10% potassium ferrocyanide (Gömöri, 2) for 15 to 30 min. This was followed by thorough washing in distilled water and subsequently in 95% alcohol over a period of 5 to 10 hr., using several changes until the staining mixture was completely eliminated. Then the gland was freed from extraneous tissue under the microscope using metal instruments. Dehydration was continued by immersion for two hours or more each, in absolute alcohol, oil of origanum, and xylol. The gland was then flattened by compression between two pieces of glass with a small metal paper clamp and left in xylol for an hour or so to flatten and then mounted in clarite, gum damar, or piccolyte. The results of such a procedure are shown in the figures. In the actual preparations the sharp blue green of the stained iron against the light unstained background considerably heightens the contrast over the photographic reproduction.

## Results

The following observations were made.

### *Speed and Uniformity of Penetration of Staining Fluid*

If the process of staining is followed under the microscope, it can be seen that the terminal arborizations of the glandular tree become outlined in a matter of minutes and the process is complete inside of a half hour. Longer immersion does not result in overstaining but does occasion the random precipitation of small particles of Prussian blue on the surface of the tissue. The stain quickly penetrates into the deepest recesses of the tissue mass, even when there is a considerable amount of muscle present, under which conditions the ordinary haematoxylin staining of whole mounts is difficult and uneven.

### *Ease of Dissection*

It is easy to dissect away extraneous tissue by virtue of the fact that the gland tree is sharply outlined while muscle and other nonglandular structures remain unstained. After the gland has been washed clear of excess staining fluid and brought into 95% alcohol it can be dissected under a binocular microscope using a combination of transmitted and direct light. The bleached muscle appears in sharp contrast to the stained gland and it is possible to separate the two very thoroughly. This is a special advantage in dealing with the axillary mammary glands, which are so closely interwoven with muscle.

### *Clarity of Gland Picture*

In tissues stained by this technique the only material that stains sharply is iron in the form of granules, the background taking on a light-green, transparent tint, due, no doubt, to traces of soluble iron in the tissue fluid. The clearing process leaves the iron-containing parts of the gland tree brilliantly outlined against the light background.

### *Measure of Alveolar Development*

The iron in the nonlactating gland of the mature female mouse is stored mainly in the alveoli and smaller ducts leading from them. As a result the stainable iron is a valuable means of assessing the degree of alveolar development. Fig. 1 shows a resting gland of C57 Black female about 60 weeks of age. The alveoli are small but distinct. Fig. 2 shows a similarly resting gland in a dba mouse of about 50 weeks of age. The difference in alveolar development is readily apparent. A study was made of the mammary glands of 50 mice of each of the following strains: C57 Black (having a very low incidence of spontaneous mammary adenocarcinoma); dba (high incidence); C3H (high incidence). It was quite clear that the extent of alveolar development was correlated with the amount of stainable iron, and the investigation is being extended to see whether the iron accumulation can be used to elucidate the factors that affect the growth and development of alveoli.

In this connection it is interesting to note that the alveolar proliferation of the mammary gland during pregnancy is accompanied by an increased deposition of iron. Fig. 3 shows this increase in iron in the gland in an early stage of pregnancy, while Fig. 4 illustrates an even greater accumulation in a more advanced stage. As Schultz (5) has pointed out, just before the termination of pregnancy and during lactation the iron either disappears or is restricted to a few areas that take the stain diffusely.

Fig. 5 shows a hyperplastic nodule. These nodules have been pointed out by many workers as occurring frequently in strains of mice with a high incidence of spontaneous cancer. By the use of the iron technique, it can be seen that such hyperplastic cells, like the normal ones, accumulate iron, and show up well in the whole mounts stained for iron.

### **Discussion**

It has been pointed out in an earlier paper (4) that the mammary glands of the mouse may be closely related to the apocrine sweat glands found in many animals, including man (1), and that the iron content of the mouse gland may be evidence of that relationship. The present study shows that the iron may be used to effect a quick and efficient whole mount stain and that, since the iron content of the resting gland can mirror the alveolar development, such a technique offers definite possibilities for the study of factors affecting the alveoli of the mammary gland. It is difficult to see what purpose such a wholesale piling up of iron might have in the normal functioning of the gland. The significance of the disappearance of the iron during lactation is also obscure. There is evidence in the blue staining of the material in the lumina of the ducts that the iron passes into the milk secretion in the early stages of lactation. As pointed out by Schultz (5) the iron starts to reappear towards the end of lactation and returns in quantity within a week when lactation ceases either in the normal course of events or as a result of weaning the young.

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### EXPLANATION OF FIGURES

FIG. 1. Photograph X30 of whole mount of a resting mammary gland of a C57 Black female mouse about 60 weeks of age; stained with Gömöri's potassium ferrocyanide - hydrochloric acid mixture. In the actual preparation the color differentiation considerably heightens the contrast.

FIG. 2. Resting gland of a dba female about 50 weeks of age; same treatment and magnification as Fig. 1; more profuse alveolar development shown up by iron stain.

FIG. 3. Gland of C3H mouse about 25 weeks of age and in early stage of pregnancy; same treatment and magnification as previous figures; shows increase in iron as alveoli develop.

FIG. 4. Gland of C3H mouse about 25 weeks of age and in later stage of pregnancy; same treatment and magnification as before; shows still further increase in alveolar and iron development.

FIG. 5. Gland of a dba mouse about 60 weeks of age; same treatment and magnification as before; shows hyperplastic nodule outlined by the iron stain.

PLATE I





# THE MULTIPLICATION OF INSECT VIRUSES AS ORGANISMS<sup>1</sup>

By G. H. BERGOLD<sup>2</sup>

## Abstract

Electron micrographs of purified preparations of four different insect viruses indicate the presence of morphologically different forms, which are probably stages of multiplication. The virus first appears as a minute spherical body. This body increases in size and the virus appears as an elongated, curved body, surrounded by a membrane. Later the virus particle straightens out, ruptures the membrane, and appears as a rod-shaped particle characteristic of insect viruses. One may assume that the rod-shaped virus particle contains several smaller subunits each of which develops into a rod. The complicated nature of multiplication indicates that insect viruses are organisms with a relatively simple-morphological structure of the mature rod.

## Introduction

The larvae of many insects are attacked by virus diseases. Two types of polyhedral diseases (2-7, 9-13, 17) and one type of capsule disease (8, 16, 18) have been described.

The polyhedral diseases are so called because of the formation of inclusion bodies, from 1 to 10  $\mu$  in diameter, which develop in large numbers in the nuclei of susceptible cells. They are highly refractive, have different and often regular shapes with sharp corners, and consist of protein. In the first type of polyhedral disease i.e. viruses affecting the larvae of susceptible Lepidoptera, polyhedral bodies develop in most tissues with the exception of the gut, the gonads, and the Malpighian tubules (4). In the other type, studied in the larva of the hymenopteran, *Gilpinia hercyniae* (Htg.), the polyhedral bodies develop exclusively in the midgut cells (10-13).

In the capsule disease (8) numerous oval-shaped inclusions are formed, chiefly in the cytoplasm. They consist of protein and are much smaller than polyhedral bodies, measuring about 0.36 by 0.23  $\mu$  in the larva of the tortricid *Cacoecia murinana* (Hb.). The nature of the capsules and of the polyhedral bodies from lepidopterous larvae has been studied intensively by physical, chemical, biological, and serological methods (5-9).

The polyhedral bodies and capsules are soluble only in weak alkalies or acids. The alkaline dissolution was chosen for experiments, as the susceptible larvae have an alkaline reaction in the alimentary tract. It was found by studies with the ultracentrifuge and by diffusion measurements that the polyhedral bodies and the capsules consist predominantly of a very homogeneous, noninfectious protein with a molecular weight of 275,000-375,000, depending on the insect species. The infectious virus agent is occluded in the polyhedral bodies and accounts for only a small percentage of their total weight.

<sup>1</sup> Manuscript received August 10, 1949.

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It can be liberated from the polyhedral bodies and the capsules under certain hydrogen ion and salt concentrations. The infectious virus particles are rod-shaped, suspensible in water, and have a high content of desoxyribonucleic acid. The different viruses have somewhat different dimensions: in the gypsy moth, *Porthezia dispar* (L.), 360 by 41 m $\mu$ ; in the silkworm, *Bombyx mori* (L.), 288 by 40 m $\mu$ ; in the spruce budworm, *Choristoneura fumiferana* (Clem.), 260 by 28 m $\mu$ ;\* and in the capsule disease of *Cacoecia murinana* (Hb.), 262 by 50 m $\mu$ .\* There are many virus rods in one polyhedral body but there is only one virus rod in one capsule.

The sedimentation curves of purified virus suspensions obtained in the ultracentrifuge show, in the case of the gypsy moth virus, three different fast-sedimenting components. The silkworm virus and the capsule virus have only one, but a rather wide, sedimentation curve. This means that the virus particles are not homogeneous in size. Early electron micrographs (5) confirmed this assumption. Later and better pictures indicated that some of the virus particles are bundles of two or four single rods (7), which fact explains the nonhomogeneity of the sedimentation curve. Fractionation and tests indicated that the virus activity is associated with the single rods. The highest activity obtained was  $4 \times 10^{-13}$  gm. protein per larva in the silkworm (7). Also observed were peculiar spherical forms and membranelike structures that could not be interpreted (5, 7). The bundle arrangement and the cross structure of the rods were thought to be possibly connected with the multiplication process.

### Material and Methods

The virus suspensions were prepared by a method that has been previously described (5-9). Very well purified, dried polyhedral bodies of pure white appearance were the starting material for the virus preparations from the gypsy moth and the silkworm, and purified capsules were used in the case of *Cacoecia murinana*. The material from the spruce budworm was not so pure, but all insoluble impurities were sedimented in five minutes, after alkaline dissolution of the polyhedral bodies, at 6500 r.p.m. (4200g). The virus was sedimented for one hour at 11,000 r.p.m. (12,000g). The clear supernatant of the dissolved polyhedral and capsule protein was discarded, and the virus sediment was suspended in distilled water and again sedimented for one hour at 11,000 r.p.m. The clear supernatant was discarded and the virus sediment suspended and diluted with distilled water to the desired concentration for the electron microscope. Collodion films were used and lowered from a carefully cleaned water surface onto the screens.

Various fixation methods were tried, but the exposure of the small droplets of virus solution on the screens for 10 to 30 min. to vapors of a 2% *osmium tetroxide* solution appeared to be the best (1). No shadow-casting was applied.

\* These figures have to be checked on a greater number of particles.

The work was carried out with a carefully prepared R.C.A. 50 kv. electron microscope, Type EMU. The instrument was supplied with an objective aperture of about  $35 \mu$  in diameter, a self-bias gun, and a telescopic viewing device. All pictures were taken at Step 3 at a magnification of 9500. The exposure time appeared to be important and was measured with a selenium photocell attached on the outside of a side window and a galvanometer with a sensitivity of  $5 \times 10^{-10} \text{ A}$  per mm. Kodak Spectroscopic plates, Type IV-O, were used. The intensity was kept as low as possible and adjusted with the galvanometer to give an exposure time of three seconds. Focusing was done at the same intensity. The best resolution obtained of the protein material was about 50 Å.

D 11 was used for the development of the negatives, contrast plates and D 72 for the positive contact process. For the negative paper enlargements, Kodabromide F 4 and D 72 were used. The photographic magnification was about five times (from 9500 up to 50,000). This method produces good detail of internal structures, but the outside membranes do not show up so well as on a white background.

### Results

Extensive observations with the electron microscope of different preparations of four different viruses led to the discovery of a "life cycle" and of multiplication forms of insect viruses. These are most evident in the polyhedral virus of the silkworm but can be seen also in the other three viruses.

Five stages of the "life-cycle" can be observed. They are summarized in Table I and are shown in Figs. 1 to 98. Figs. 32 and 33, 49, 79 and 80, 97, and 98 are typical pictures showing different stages of development of virus suspensions from *Bombyx mori*, *Porthetria dispar*, *Choristoneura fumiferana*, and *Cacoecia murinana*.

TABLE I  
DESCRIPTIONS OF THE DEVELOPING STAGES OF INSECT VIRUSES

Polyhedral viruses		Capsule virus	
<i>Bombyx mori</i>	<i>Choristoneura fumiferana</i>	<i>Porthetria dispar</i>	<i>Cacoecia murinana</i>

#### Stage I

Somewhat spherical forms developing from unknown size

200 m $\mu$	300 m $\mu$	300 m $\mu$	150 m $\mu$
Elongated "germ" becoming visible in a spherical membrane			
Some differentiation. Figs. 1 - 6	Some differentiation. Figs. 34 - 38	Clear differentiation. Germ wider. Figs. 51 - 57	Clear differentiation. Figs. 81 - 84

TABLE I—Concluded

## DESCRIPTION OF THE DEVELOPING STAGES OF INSECT VIRUSES—Concluded

Polyhedral viruses			Capsule virus
<i>Bombyx mori</i>	<i>Choristoneura fumiferana</i>	<i>Porthetria dispar</i>	<i>Cacoecia murinana</i>
Stage II			
"Germ" elongating bending to a curved shape inside of the membrane			
Figs. 7–10	Figs. 39–40	Figs. 58–60	Figs. 85–87
Stage III			
Curve-shaped "germ" straightens out to a more rod-shaped particle (still in membrane)			
Figs. 11–14	Fig. 41	Figs. 61–62	Figs. 88–91 Double forms visible
Stage IV			
Shrinking of the germ and decrease in width (still in membrane)			
Heavy structural changes. Figs. 15–21	Sometimes dividing lengthwise. Figs. 42–44	Heavy structural changes. Dividing lengthwise. Figs. 63–73	Stage IV No shrinking could be observed
Stage V			
Rod-shaped particle leaving spherical membrane			
Second, tube-shaped membrane Figs. 22–24	Fig. 45	Splitting frequently in four rods. Figs. 74–76	Fig. 92
Empty membranes Figs. 25–27			
Final rod-shaped, smooth particle Figs. 28–29	Figs. 48–50	Final rod-shaped, smooth particle. Figs. 77–78	Final wider rod, slightly bent; with structures. Figs. 93–96
Sometimes showing groove lengthwise Figs. 30–31	Figs. 46–47		

## Discussion

The development of the polyhedral bodies is a very rapid process. It is therefore, probable that virus particles in different stages of their development become suddenly occluded and that they may be liberated by dissolution

The only other particles that could be occluded by the polyhedral bodies are nuclear components of the host cell. Numerous dissolutions of different polyhedral bodies always show the same forms. This would not result if any nuclear components become occluded.

The next important point is to prove the connection between the infectious rod-shaped particles (Figs. 28 to 31) and the spherical forms. This is demonstrated by Figs. 22 to 24, which show rod-shaped particles that have just emerged from a spherical membrane. Earlier stages (Figs. 4 to 21) also show that the particle is surrounded by a membrane. This membrane returns somewhat to its original spherical form (Figs. 4, 6, 8) when the virus particle escapes (Figs. 22 to 24). The presence of this spherical membrane is therefore the proof of a connection between the virus rods and the spherical developing stages. The early stages (Figs. 1 to 3, 34 to 36, 51 to 52) are not convincing. But one may be able to fractionate and concentrate these and probably also even earlier stages. It seems unlikely (6) that the reported particles of 10 m $\mu$  diameter (2, 4, 15) are the initial stages.

Also, Figs. 22 to 26 and 45 show a tube-shaped membrane of the same dimensions as the rods. It appears therefore that the rods are surrounded in the developing stages by two membranes. It is probable that the particles observed in Figs. 22 to 24 were in an advanced stage of development when occluded by the polyhedral protein. The emergence of the rods certainly takes place while the rods are drying out on the film in the high vacuum. Both membranes must consist of a very elastic substance. Figs. 25 to 27 show that the membranes are not artifacts since they break in a characteristic way and have the appearance of a broken rubber ball with curled edges. So far it is uncertain whether the rod leaves the second, tube-shaped membrane under natural conditions.

There is, as yet, no explanation of how the rod-shaped particles begin to multiply again. Two possibilities are suggested: either the rod-shaped particle shrinks enormously to a small sphere, or it contains several small subunits. The first possibility is very unlikely. But the second can be considered, and may be confirmed by the following. If only one rod develops from one, no multiplication takes place at all. In the case of the silkworm polyhedral virus, it is not known whether the rods (Figs. 30, 31) showing lengthwise grooves really split. Furthermore, these double rods are rather rare. The rapid increase of virus particles could hardly be explained, if this were the only kind of multiplication. It is therefore probable that a single rod contains smaller subunits, which can develop into rods. The possibility of the existence of such subunits, based on the serological results of parallel mutations of different strains (14), has been discussed even in the case of tobacco mosaic virus.

The complicated nature of development and of multiplication described above certainly indicates that insect viruses are organisms with spherical developing stages and a rod-shaped stage of relatively simple morphological structure.

### Acknowledgment

The author would like to thank F. T. Bird for discussing the problem and for his help in preparing this paper.

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### EXPLANATION OF PLATES

### PLATE I

FIGS. 1 TO 33. *The developing stages of the polyhedral virus of the silkworm, Bombyx mori (L.). Magnification 42,500X.*

FIGS. 1 TO 6. *Stage I. Spherical forms showing the "germ" within a membrane.*

FIGS. 7 TO 10. *Stage II. "Germ" elongating and seen in a curved shape within a membrane.*

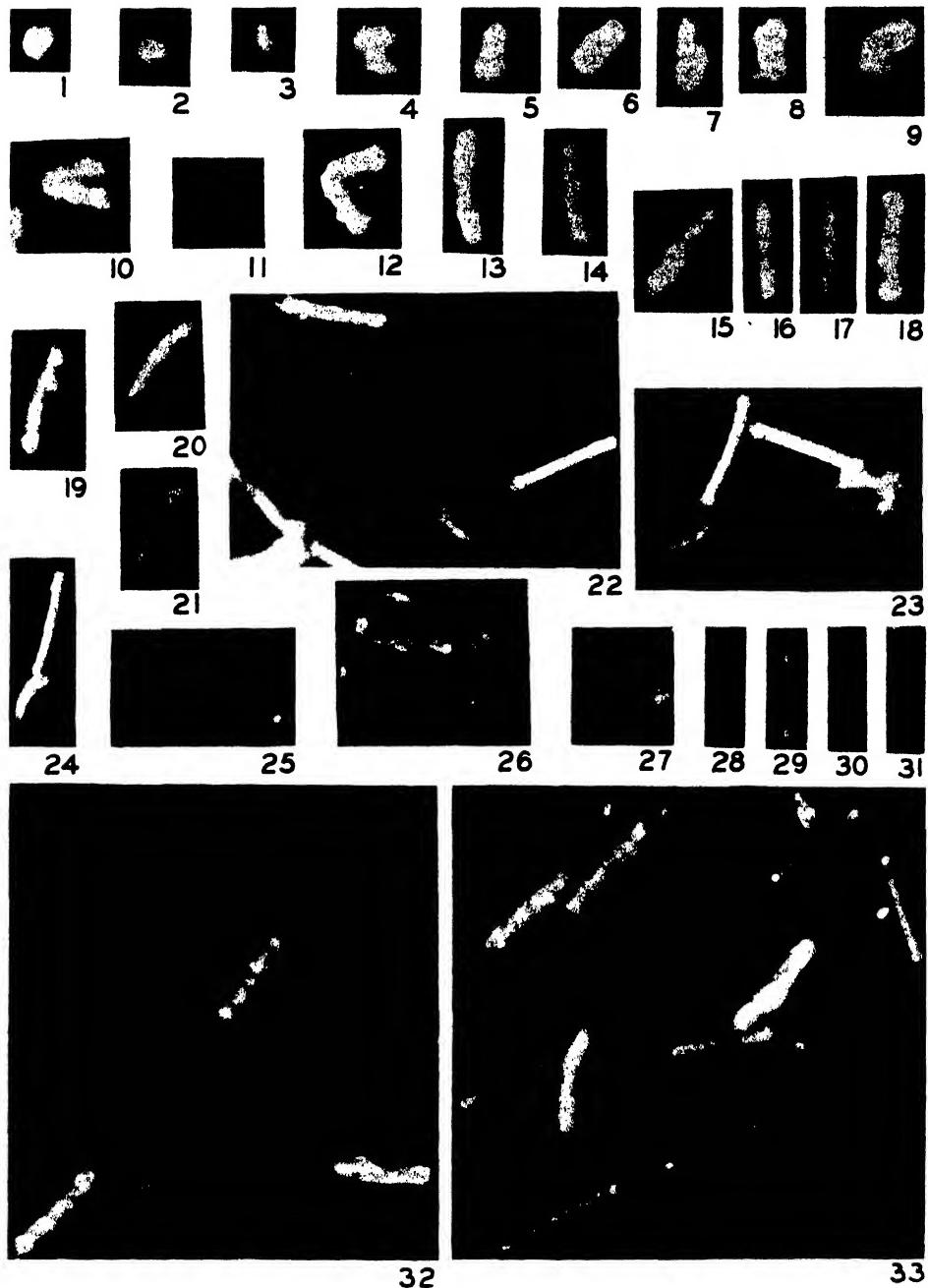
FIGS. 11 TO 14. *Stage III. "Germ" straightening out to a rod-shaped form. Still in the membrane.*

FIGS. 15 TO 21. *Stage IV. Shrinking of "germ" and decreasing in width. Heavy structural changes. Still in membrane.*

FIGS. 22 TO 31. *Stage V. Rod-shaped particles leaving spherical and tube-shaped membranes. Figs. 22 to 24; Figs. 25 to 27: empty membranes; Figs. 28, 29: single, rod-shaped, smooth virus particles; FIGS. 30, 31: rod-shaped virus particles showing groove lengthwise.*

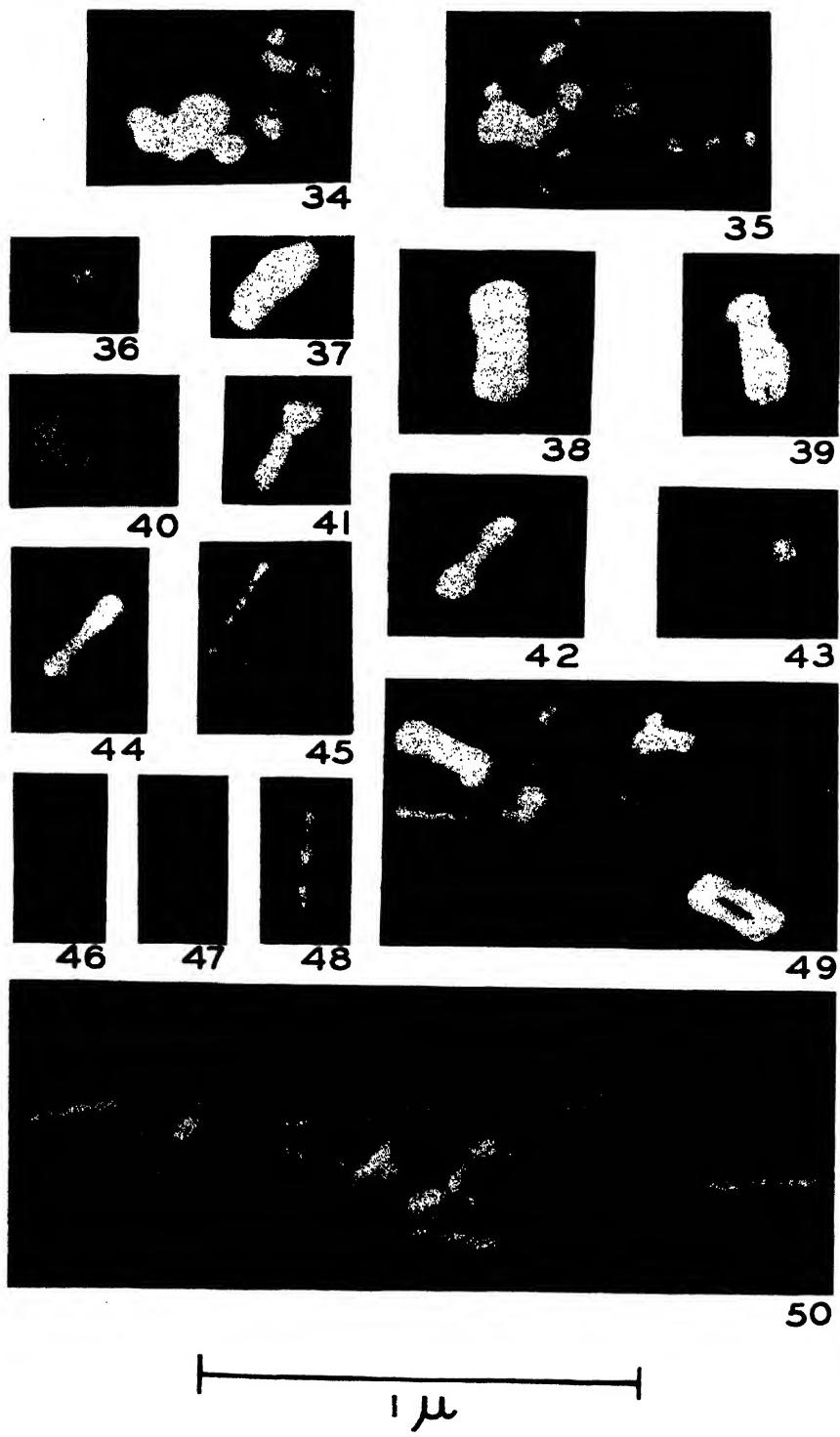
FIGS. 32, 33. *Typical pictures of virus suspensions from the silkworm, showing different stages of development.*

PLATE I



1  $\mu$

PLATE II



## PLATE II

FIGS. 34 TO 50. *The developing stages of the polyhedral virus of the spruce budworm, Choristoneura fumiferana (Clem.). Magnification 50,000X.*

FIGS. 34 TO 38. *Stage I. Spherical forms showing "germ" within a membrane.*

FIGS. 39, 40. *Stage II. "Germ" elongating and seen in a curved shape within a membrane.*

FIG. 41. *Stage III. "Germ" straightening out to a rod-shaped form. Still in membrane.*

FIGS. 42 TO 44. *Stage IV. Shrinking of "germ" and decreasing in width. Still in membrane. Sometimes dividing lengthwise. Fig. 43.*

FIGS. 45 TO 48, 50. *Stage V. Rod-shaped particles leaving spherical and tube-shaped membranes, Fig. 45; Figs. 46-48, 50: single, rod-shaped, smooth particles; Fig. 48: rod-shaped particle with cross structures; Fig. 47: rod-shaped particle showing groove lengthwise.*

FIG. 49. *Typical picture of virus suspension from the spruce budworm, showing different stages of development.*

## PLATE III

FIGS. 51 TO 80. *The developing stages of the polyhedral virus of the gypsy moth, Porthetria dispar (L.). Magnification 42,500X.*

FIGS. 51 TO 57. *Stage I. Spherical forms showing broad "germ" with structures within the membrane.*

FIGS. 58 TO 60. *Stage II. "Germ" elongating and seen in a curved shape within a membrane.*

FIGS. 61, 62. *Stage III. "Germ" straightening out to a rod-shaped form. Still in the membrane.*

FIGS. 63 TO 73. *Stage IV. Shrinking of "germ" and decreasing in width. Heavy structural changes. Sometimes lengthwise divisions visible.*

FIGS. 74 TO 78. *Stage V. Rod-shaped particles leaving spherical membranes. Figs. 74, 75; Figs. 74 to 76: splitting into four single rods; Figs. 77, 78: single, rod-shaped, smooth virus particles.*

FIGS. 79, 80. *Typical pictures of virus suspensions from the gypsy moth, showing different stages of development.*

## PLATE IV

FIGS. 81 TO 98. *The developing stages of the capsule virus of Cacoecia murinana (Hb.). Magnification 50,000X.*

FIGS. 81 TO 84. *Stage I. Spherical forms showing the "germ" with structures within the membrane.*

FIGS. 85 TO 87. *Stage II. "Germ" elongating and seen in a curved shape within a membrane.*

FIGS. 88 TO 91. *Stage III. "Germ" straightening out to a rod-shaped form. Double forms visible. Still in the membrane.*

FIGS. 92 TO 96. *Stage V. Rod-shaped particle leaving spherical membrane. Fig. 92; Figs. 93 to 96: single rod-shaped particles broad, slightly bent, with some structures; sometimes in spiral form (Fig. 93).*

FIGS. 97, 98. *Typical pictures of virus suspensions from Cacoecia murinana, showing different stages of development.*

## THE PYRIDINE NUCLEOTIDE CONTENT OF HUMAN BLOOD CELLS IN ANEMIA<sup>1</sup>

By M. C. BLANCHAER

### Abstract

The pyridine nucleotide content of the blood cells was measured in five normal individuals and in 27 hospital patients with various degrees of anemia. The pyridine nucleotide values of 12 anemic patients who had consumed an adequate diet for some time prior to the study were higher than those of the normal subjects. A negative correlation was observed between the cellular pyridine nucleotide content and the severity of the anemia when the latter was expressed as the logarithm of either the red cell count or the hemoglobin concentration. The remaining 15 patients had dietary histories suggestive of a low intake of niacin and protein. The blood cell pyridine nucleotide levels of this group were of the same order as those of the normal subjects but in most cases were distinctly below those of the well nourished anemic patients with a comparable degree of anemia.

### Introduction

The realization that nicotinamide is an essential component of the molecule of the two pyridine nucleotides (PN) has led to a number of studies (1, 2, 4, 5, 11, 12) on the relationship of niacin status in man to the pyridine nucleotide content of the blood cells. The neglect of factors other than niacin intake influencing such measurements (5) may have given rise to the conflicting evidence presented in these reports. Vilter *et al.* (11) suggested that anemia per se may affect the PN concentration in the blood cells, since such values were frequently found to be elevated in anemic individuals. Indirect confirmation of this observation may be found in the data of Melnick *et al.* (7) who reported an increased nicotinic acid concentration in the blood cells in anemia.

The present investigation was undertaken to determine the nature of the relationship between anemia and the PN content of the blood cells.

### Methods

The subjects consisted of five apparently healthy laboratory workers and 27 hospital patients representing the commoner types of primary and secondary anemia seen in this region. The subjects were divided into two groups on the basis of nutritional status. Group A consisted of the five normal laboratory workers and 12 patients judged to have consumed adequate amounts of a normal diet for some time prior to the study. The remaining patients were assigned to Group B. Most of these had dietary histories suggestive of a low intake of niacin and protein and many suffered from severe illnesses accompanied by general malnutrition, but none showed overt clinical signs of vitamin deficiency.

<sup>1</sup> Manuscript received October 29, 1949.

Contribution from the Nutrition Laboratory, Department of Physiology and Medical Research, University of Manitoba, and the Winnipeg General Hospital, Winnipeg, Man.

Blood specimens were obtained by venipuncture approximately three hours after breakfast. The anticoagulant was a dried mixture of ammonium and potassium oxalate (6). Methods described by Wintrobe (13) were used in determining hematocrit values and counting red cells, leucocytes, and reticulocytes. Haemoglobin was estimated by the alkaline hematin method of Clegg and King (3). The PN concentration of each whole blood specimen was determined in duplicate by the fluorometric procedure of Levitas *et al.* (6). Since previous workers (8) using specific biological methods have shown that the pyridine nucleotides in blood are limited to the cells, the whole blood values were corrected for the apparent PN content of the plasma measured by the same fluorometric procedure. No evidence is available to indicate whether the substance in plasma that behaves like the pyridine nucleotides in the fluorometric procedure is limited to the plasma or is present in both plasma and cells. In calculating the results, it was assumed that this substance was limited to the plasma. The corrected values were not markedly different when the alternative assumption was made. The corrected PN levels were assumed to be mainly dependent upon the red cell PN content since it was observed that moderate variations in the leucocyte count had little influence on the values.

Results obtained using an internal standard of N<sup>1</sup>-methylnicotinamide agreed closely with those calculated from external standards, suggesting that extraneous material in the samples had little quenching effect on the fluorescence. The average difference between the results of duplicate estimations on 20 specimens of whole blood was 6.4% (S.D.  $\pm$  4.2). The percentage difference between duplicates showed no tendency to vary with the hemoglobin or PN content of the specimens.

Since the degree of anemia may be measured in terms of the hematocrit value, hemoglobin concentration, or red cell count, the PN content of the blood cells was expressed in corresponding units, i.e., micrograms per milliliter cells (PN/ml. cells), micrograms per gram haemoglobin (PN/Hb), and micrograms per billion (10<sup>9</sup>) red cells (PN/RBC). The method of calculating the results was as follows:

$$\left. \begin{aligned} \text{Corrected PN/ml.} \\ \text{whole blood} \end{aligned} \right\} = (\mu\text{gm. PN/ml. whole blood}) \\ - \left( \mu\text{gm. PN/ml. plasma} \times \frac{100 - \text{hematocrit}}{100} \right)$$

$$\text{PN/ml. cells} = \frac{\text{Corrected PN/ml. whole blood} \times 100}{\text{Hematocrit}}$$

$$\text{PN/RBC} = \frac{\text{Corrected PN/ml. whole blood}}{\text{Red cells/cu. mm.} \times 10^{-6}}$$

$$\text{PN/Hb} = \frac{\text{Corrected PN/ml. whole blood}}{\text{Gm. hemoglobin/ml. whole blood}}$$

In an attempt to simulate the effect on the method of some of the chemical and physical differences between blood of normal and anemic subjects, the following experiments were performed. Analyses were made on the blood of two normal individuals and compared with the results obtained after dilution with homologous plasma. It may be seen in Table I that addition of plasma to blood had little effect on the final cellular PN values when appropriate corrections were made for dilution and the plasma blank.

TABLE I  
THE EFFECT OF DILUTION ON THE ESTIMATION OF PN IN BLOOD CELLS

Subject	Specimen	Hematocrit	Haemoglobin, gm. %	PN*, $\mu\text{gm./ml.}$	PN/ml. cells	PN/Hb
1	Plasma	—	—	1.4 2.2	—	—
	Blood	47.5	15.8	37.2 39.5	79.0	237
	Blood - plasma	23.6	8.2	20.2 20.2	79.7	229
2	Plasma	—	—	1.0	—	—
	Blood	41.9	13.2	26.2 25.6	60.0	192
	Blood - plasma	16.6	5.6	10.8 11.9	63.9	189

\* Duplicates, uncorrected for plasma blank.

### Results

The results obtained in Group A, which consisted of those subjects who had consumed an adequate diet, are shown in Table II. While the whole blood PN levels of the anemic patients were lower than those of the normal subjects, the cellular PN values were higher. The PN/RBC and PN/Hb values of Group A were found to be negatively related to the logarithm of the red cell count and hemoglobin concentration respectively, as shown in Figs. 1 and 2. A similar but less exact relationship existed between the PN/ml. cells and the hematocrit readings.

In contrast to the findings in Group A, the whole blood PN levels of the subjects in Group B, shown in Table III, decreased roughly in proportion to the severity of the anemia. The cellular PN values were of the same order as those of the normal subjects in Group A or slightly greater. However, as shown in Figs. 1 and 2, the PN/RBC and PN/Hb values with few exceptions, were lower than those of the patients in Group A with comparable degrees of anemia.

TABLE II  
DATA ON SUBJECTS IN GROUP A

No.	Sex	Age	W.B.C. per cu. mm.	R.B.C. millions per cu. mm.	Haemo- globin, gm. %	Reticulo- cytes, %	Hemato- crit	Pyridine nucleotides μgm./ml.			PN/RBC	Diagnosis
								Plasma	Whole blood*	Cells		
1	M	28	7500	16.4	—	48.5	1.6	35.8	72.1	213	—	Normal
2	M	21	—	15.6	—	48.3	1.2	35.5	80.0	194	—	Normal
3	M	24	—	15.0	—	44.1	0.3	29.4	66.7	196	—	Normal
4	M	65	9500	5.42	14.1	48.5	1.4	31.5	63.5	218	5.68	Normal
5	F	74	6200	5.07	13.7	—	43.6	2.0	33.3	73.8	235	Bunion
6	F	23	3600	4.92	13.4	—	42.6	2.2	28.5	64.8	206	Normal
7	F	48	2800	5.05	12.7	0.4	43.0	0.9	31.8	72.3	246	6.20
8	M	61	4320	4.74	12.0	—	43.2	1.9	32.5	72.7	262	Early gastric carcinoma; normal appetite
9	F	66	4100	3.90	11.6	—	38.2	1.2	32.8	84.0	276	Psychoneurosis
10	F	18	7700	3.20	8.9	4.4	29.4	2.2	32.2	104.2	344	Abortion with hemorrhage
11	F	67	11,350	3.37	8.4	2.0	27.4	0.7	26.3	94.0	306	Lung abscess**
12	F	30	10,300	4.35	7.7	1.2	30.3	2.3	31.7	97.2	392	Iron deficiency anemia
13	F	19	4100	3.24	7.6	0.6	27.0	1.4	25.8	91.4	324	Duodenal ulcer
14	M	64	8800	2.12	6.9	18.0	25.1	1.7	27.6	104.8	381	Pernicious anemia; after liver extract
15	F	43	7100	3.85	6.3	1.2	24.2	1.0	20.9	83.1	318	Iron deficiency anemia
16	F	74	4300	1.81	6.0	18.7	20.4	1.3	21.5	100.0	340	Pernicious anemia; after liver extract
17	M	72	6900	3.42	5.2	—	23.7	1.9	24.4	97.1	442	Bleeding from esophageal diver- ticulum
											6.73	

\*Uncorrected for plasma blank activity.

\*\* After 210 mgm. nicotinamide in five days.

In neither group was a correlation found between the cellular PN levels and age, sex, plasma protein concentration, leucocyte count, mean corpuscular

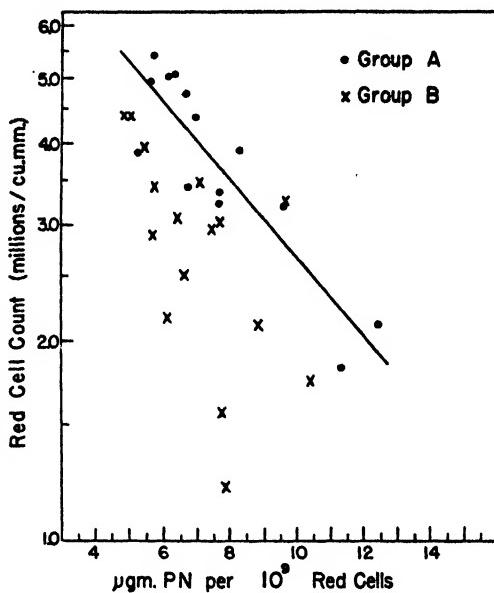


FIG. 1. The relationship between blood cell PN/RBC values and the logarithm of the whole blood red cell count. The regression line for Group A is shown.

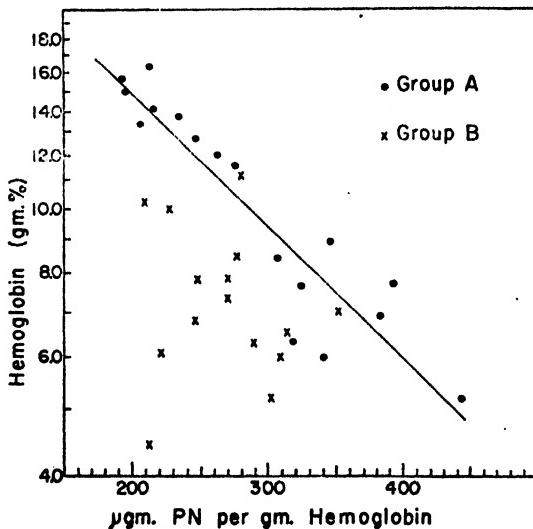


FIG. 2. The relationship between blood cell PN/Hb values and the logarithm of the whole blood haemoglobin concentration. The regression line for Group A is shown.

volume, or mean corpuscular haemoglobin concentration. The 16 reticulocyte counts available in the present study were considered too few to permit an

TABLE III  
DATA ON SUBJECTS IN GROUP B

No.	Sex	Age	W.B.C. per cu. mm.	R.B.C. millions per cu. mm.	Hemo- globin, gm. %	Reticulo- cytes, %	Hemato- crit	Pyridine nucleotides			PN/RBC	Diagnosis
								Plasma	Whole blood*	Cells		
18	M	67	5500	3.25	11.2	—	37.6	1.2	32.6	83.6	280	9.65 Gastrocolic fistula
19	F	45	6300	4.38	10.2	0.6	34.6	1.0	22.1	61.9	210	4.89 Hemorrhage; duodenal ulcer
20	F	88	8400	4.36	10.0	—	34.1	1.4	23.7	67.0	228	5.24 Senile osteoarthritis
21	M	65	4900	3.03	8.5	—	30.2	3.3	25.8	77.9	277	7.75 Bronchopneumonia
22	F	42	12,300	3.40	7.9	1.2	29.4	1.2	20.4	66.8	248	5.77 Lung abscess
23	M	67	8800	3.92	7.9	1.0	28.0	1.1	22.1	76.1	270	5.43 Hemorrhage; duodenal ulcer
24	M	76	5620	3.07	7.3	—	24.2	1.9	21.2	81.4	270	6.41 Diabetes; malnutrition
25	F	87	4200	3.48	7.0	—	28.7	2.0	26.2	86.4	353	7.14 Senile osteoarthritis
26	M	75	3900	2.95	7.0	0.4	26.4	1.4	23.5	83.5	314	7.48 Hemorrhage; gastric carcinoma
27	M	82	10,800	2.52	6.8	1.6	22.5	1.3	17.8	74.7	246	6.67 Hemorrhage; gastric carcinoma
28	F	75	1550	1.74	6.3	0.6	21.0	1.0	19.0	86.8	290	10.48 Pernicious anemia; with diarrhea
29	F	71	3700	2.17	6.1	3.6	20.2	0.9	14.1	66.3	221	6.17 Ovarian carcinoma; metastases; anorexia
30	M	80	1500	2.09	6.0	—	20.1	1.4	19.6	91.9	310	8.82 Panhypoplastic anemia
31	M	59	8650	2.88	5.2	1.2	22.9	0.9	16.5	68.2	302	5.69 Malfunction of gastroenterostomy
32	M	65	1600	1.20	4.4	0.5	14.8	1.4	10.6	63.9	213	7.89 Sprue; scurvy

\* Uncorrected for plasma blank activity.

adequate assessment of the influence of an increased number of immature red cells on the cellular PN values.

### Discussion

The observations described above suggest that the blood cell PN levels of malnourished anemic subjects cannot be satisfactorily compared with those of normal individuals. It was only when the values of the poorly nourished patients were compared with those of well nourished patients with a similar degree of anemia that a relationship between general nutritional status and the cellular PN levels became apparent. Further observations using more refined methods for assessing niacin status are required before the relationship of blood cell PN values to human niacin sufficiency can be determined.

Because of the apparently normal PN content of the blood cells in pellagra (1, 2, 5), it has been inferred that such measurements are of no use in the detection of niacin deficiency. However, the frequent occurrence of anemia in pellagra (9, 10) would suggest that the observations may have been made in the presence of anemia. The present findings indicate that "normal" blood cell PN levels found under these conditions may reflect a state of niacin deficiency.

### Acknowledgments

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# THE STABILITY OF ASCORBIC ACID IN SOLUTION<sup>1</sup>

BY JAMES CAMPBELL AND W. G. TUBB

## Abstract

The stability of ascorbic acid in aqueous solution was increased under certain conditions by oxalic acid, metaphosphoric acid, glutathione, thiourea, and sodium diethyldithiocarbamate. A slight protective effect was exerted by creatinine; but formic, phthalic, and orthophosphoric acids, creatine, and caffeine had no demonstrable effect. In all these instances the pH, concentrations of reagents, etc., must be considered. In oxalate and thiourea maximum stability occurred at pH 2.5 to 3.0 and in glutathione at pH 3.6 to 4.2. The latter substance itself was also most stable at pH 3 to 4. At the optimum pH a concentration of 40 mM of oxalate gave maximal protection, this being independent of the initial concentration of ascorbic acid over the range 2 to 20 mM. Thus a stoichiometric relationship between the concentrations of the ascorbic acid and the oxalate required for protection was not found. A region of minimum solubility of oxalate in water occurred at pH 2.4 to 3.0, which coincides with the pH at which the maximum protective effect occurs and with the highest relative concentration of sodium hydrogen oxalate (or sodium hydrogen oxalate monohydrate). The absorption of ultraviolet light by ascorbic acid was altered by pH, the maximum shifting from 244 to 268 m $\mu$  from pH 2.8 to 4.5. The molecular extinction coefficient of ascorbic acid also changed with pH and was minimal at pH 4.0. This effect occurred in oxalate, which has a specific protective effect, and also in formate and orthophosphate, which have no specific protective effect. The possible mechanisms for the protection of ascorbic acid by oxalate are discussed.

## Introduction

It is important in the practice of nutrition that ascorbic acid is a highly labile substance. Since the vitamin is apparently maintained in the reduced form in tissues under conditions that would be expected to produce rapid oxidation in the absence of living processes, the problem of its stability is also of physiological interest. While there are many factors that influence the rate of destruction of ascorbic acid, one general aspect of the entire problem concerns the effects of accompanying, nonenzymic, organic substances. Examples of these, which are known to protect the vitamin, are glutathione, cystine, cysteine, and proteins (6, 24). It has been shown that oxalic acid has a powerful protective effect (17, 18, 25) and in the present work particular attention was given to the nature of this phenomenon. Although an array of substances has been studied, it is often difficult to compare their effects on ascorbic acid because of variations in the conditions used by different authors. We have therefore investigated the problem under comparable controlled conditions.

## Methods

The water for the preparation of solutions was distilled from a laboratory still and twice from all-glass apparatus. The redistillations lowered the copper content (tested by the method of McFarlane, 21) and improved the

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stability of the ascorbic acid solutions. The citrate-phosphate buffer of McIlvaine (0.1 *M* citric acid and 0.2 *M* disodium phosphate (Clark, 8)) when used, comprised one-half the volume of the final solutions. The pH was determined with a glass electrode (Beckman pH meter).

Ascorbic acid was determined either by titration with iodate (to 5 ml. of the unknown solutions, 1 ml. 10% potassium iodide, 2 ml. of 2 *N* sulphuric acid, and one drop of starch indicator were added and this solution was titrated with 0.01 *N* potassium iodate), or by titration of the solutions adjusted to pH 3.6 with 2,6-dichlorophenolindophenol according to Bessey and King (3). Glutathione in the presence of ascorbic acid was determined as the difference between the iodate and the dye titrations (20, 30). The former titration in the presence of glutathione was done at 50° C., as this improved the sharpness of the end points. To determine oxalate in the presence of ascorbic acid, the former was first separated by precipitation as the calcium salt by adding to 1 ml. of the slightly acid solution in a 15 ml. centrifuge tube, 5 ml. *M*/10 calcium chloride, a drop of methyl red, and ammonia solution to change the indicator to yellow. After centrifuging, the precipitate was dissolved in 2 ml. of 2 *N* sulphuric acid and was titrated with permanganate. The solutions were kept at 37° C. in a dark, electrically-controlled cabinet. At appropriate intervals samples were removed for the determination of ascorbic acid; and by plotting concentration against time, the rates of destruction were determined.

The final concentrations of the constituents of the solutions are given as millimolecular weights per liter (*mM*), except when otherwise mentioned. For example, a solution containing 0.5 gm. oxalic acid per 100 ml. is equivalent to a concentration of 55.5 *mM* oxalate; the proportion of free acid to its salts being dependent chiefly on the final pH of the solutions.

### Results

Under conditions that were nearly optimal for stability, the rate of disappearance of ascorbic acid was found to agree with a reaction of the first order, e.g., near the optimum pH for stability in the presence of oxalate, this relation was maintained up to 96 hr. The velocity constant (*k*) was obtained by the equation:

$$k = 2.303 \frac{(2 - \log c)}{t}$$

where *c* = the percentage of ascorbic acid remaining

*t* = time in hours.

Under conditions in which the ascorbic acid was unstable the first order relation was generally not found, and the results of these experiments are expressed as the percentage of ascorbic acid remaining after a certain time interval. In these cases more complicated reactions had probably occurred (Silverblatt, Robinson, and King, 27), and determinations made at shorter time intervals might have revealed a first order relation.

### *Comparison of the Stability of Ascorbic Acid in Oxalic and Metaphosphoric Acids*

In preliminary experiments the stability of 5.68 mM ascorbic acid in various concentrations of free oxalic and metaphosphoric acids was determined. Five ml. portions of the solutions were kept at 37° C. in 25 ml. test tubes, and after appropriate intervals were analyzed for ascorbic acid. The ascorbic acid was about as stable in the presence of 375 to 63 mM metaphosphoric acid (MPA) from pH 1.65 to 2.18 as in 222 and 55.5 mM oxalic acid of pH 1.18 and 1.65 (Table I). When the concentration of oxalic acid was reduced to 11.1 mM (pH 2.18) the stability was reduced. Further experiments, *vide infra*, showed that this was due to the lower concentration of oxalic acid and not to the rise in pH.

TABLE I

THE EFFECT OF METAPHOSPHORIC ACID (MPA) AND OF OXALIC ACID AND THEIR SALTS ON THE STABILITY OF ASCORBIC ACID IN SOLUTION. THE INITIAL CONCENTRATION OF ASCORBIC ACID WAS 5.68 mM; THE TEMPERATURE 37° C.

Substance added	Concentration, mM	pH	<i>k</i>
MPA	375	1.65	0.0044
MPA	188	1.88	0.0039
MPA	63	2.18	0.0044
Oxalic acid	222	1.18	0.0039
Oxalic acid	55.5	1.65	0.0039
Oxalic acid	11.1	2.18	0.0057
MPA + sodium hydroxide	188	3.05	0.0094
MPA + sodium phosphate	188	2.97	0.0094
Oxalic acid + sodium hydroxide	55.5	3.45	0.00149
Oxalic acid + sodium phosphate	55.5	3.38	0.00163

When the pH of a 188 mM solution of MPA was adjusted to  $3.0 \pm 0.05$ , the stability of ascorbic acid was less than in the free MPA (Table I). When the pH of a 55.5 mM oxalic acid solution was raised to  $3.4 \pm 0.05$ , the protective effect was greater than in the free acid and exceeded that of free MPA. The use of sodium hydroxide or sodium phosphate to adjust the pH of the oxalic acid and MPA solutions produced parallel changes, i.e., the orthophosphate ion had no effect on the stability of the ascorbic acid. The course of the reaction was more regular in the solutions whose pH had been adjusted by alkali. Probably this was due to the buffering effect produced.

### *The Optimum pH for Stability in Oxalate*

The stability of ascorbic acid (initially 5.68 mM) in the presence of 55.5 mM oxalate over the range pH 1 to 5 was determined at intervals up to 90 hr. (Fig. 1). A definite pH optimum for stability occurred at pH 2.5 to 3.0, and on either side of this optimum the rate of destruction of ascorbic acid

appeared to be equal for equal changes of pH. The rates of disappearance of ascorbic acid followed the first order reaction relationship and the least value of  $k$  (0.00075) occurred at pH 2.8.

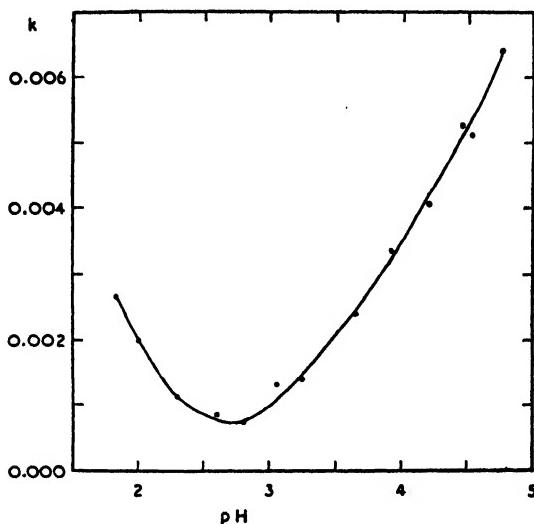


FIG. 1. *The rate of disappearance of ascorbic acid, initial concentration 5.68 mM, from solution in 55.5 mM oxalate at 37° C.*

#### *The Effect of pH on the Solubility of Oxalate*

It was observed that alteration of the pH of oxalic acid solutions by the addition of sodium hydroxide had a decided influence on the amount of solid in solution. The nature of this change was determined by adding to 25 ml. of 5% oxalic acid sufficient 1 N sodium hydroxide and water to bring to the desired pH and the final volume of 45 ml. When equilibrated at 11.6° C. by continuously rocking the containers in a water bath, solid separated out from the solutions at pH 1.32 to 3.89. Samples were withdrawn after 20 and 46 hr., by means of a pipette with a filter-tip, and the oxalate was determined by the permanganate method. From pH 2.4 to 3.0 (average pH 2.70) the amount of oxalate in solution was minimum (Fig. 2). At pH 2.41 the oxalate per ml. saturated solution was 10.55 mgm. calculated as  $C_2O_4$  (0.1199 M). When the temperature was raised to 21.5° C. the minimum solubility of oxalate occurred in the same pH range; i.e., at pH 2.41, 2.65, and 3.00 the amounts of oxalate per ml. saturated solution were 15.4, 15.1, and 15.3 mgm. Thus the range of pH over which the oxalate is least soluble is also the range at which ascorbic acid in solution with lower concentrations of oxalate is most stable.

In solutions similar to the above, but which contained also 5.68 mM ascorbic acid, it was found that the added ascorbic acid had little, if any, effect on the solubility of the oxalate under these conditions.

The data of Foote and Andrew (9) on the ternary system sodium oxalate - oxalic acid - water at 25° C. show that the least concentration of oxalate radical in a saturated solution occurs when the saturating solid is sodium

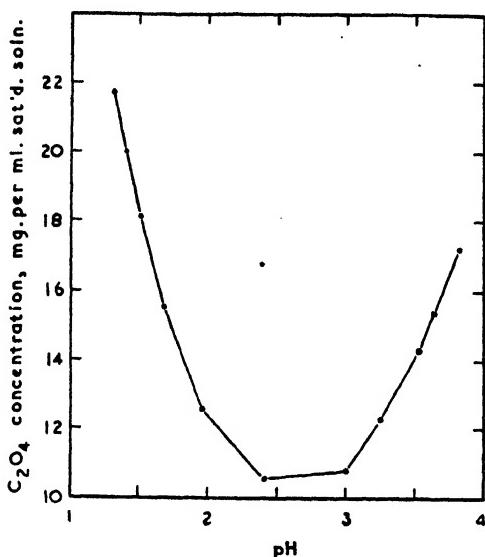


FIG. 2. *The effect of pH on the solubility of oxalate, calculated as  $C_2O_4$ , in water at 11.6° C.*

hydrogen oxalate monohydrate and when the composition of the saturated solution can be described in terms of sodium hydrogen oxalate (or sodium hydrogen oxalate monohydrate) and water only, i.e., the system is binary. From our data this least oxalate concentration occurs at pH 2.70.

The dissociation constants of oxalic acid are 1.19 and 4.22. It can be calculated from the Henderson-Hasselbach equation that at pH 2.70 (the mid-point of the range of least solubility of oxalate in mixtures of oxalic acid and sodium oxalate) the ratio oxalic acid/sodium hydrogen oxalate/disodium oxalate is 1/32/1, and that the relative amount of sodium hydrogen oxalate is greatest. In view of the assumptions involved, these calculated values may have limited application. It may be concluded that at the pH of the greatest stability of ascorbic acid in oxalate, the latter is preponderantly in the form sodium hydrogen oxalate (or sodium hydrogen oxalate monohydrate).

#### *The Concentration of Oxalate and the Stability of Ascorbic Acid*

The effect of various concentrations of oxalate on the stability of 5.68 mM ascorbic acid was determined at the optimum pH of 2.8 and in the presence of citrate-phosphate buffer, which comprised 50% of the final volume (Table II, Part b). The pH of the solutions did not change appreciably during 48 hr. of incubation. The curves drawn from the data showed that as the concentration of oxalate increased, the protective effect increased to a maximum at about 40 mM oxalate and above, giving a value of  $k = 0.0006$ . Below this

TABLE II

EFFECT OF VARIOUS CONCENTRATIONS OF OXALATE ON THE STABILITY OF ASCORBIC ACID IN THE PRESENCE OF CITRATE-PHOSPHATE BUFFER AT pH 2.8 AND AT 37° C.

Ascorbic acid initial concentration, mM	Concentration of oxalate, mM	pH	<i>k</i>
20	111	2.70	0.000642
	55.5	2.76	0.000936
	27.5	2.78	0.00125
	11.1	2.80	0.00311
	5.55	2.81	0.00415
	2.22	2.81	0.00592
	1.11	2.82	0.00791
	0.0	3.18	0.0206
5.68	111	2.76	0.000601
	55.5	2.77	0.000601
	27.5	2.78	0.00147
	11.1	2.78	0.00346
	5.55	2.80	0.00823
	2.22	2.80	0.0138
	1.11	2.80	0.0146
	0	2.80	0.0206
2.0	111	2.70	0.00121
	55.5	2.76	0.00151
	27.5	2.78	0.00192
	11.1	2.82	0.00521
	5.55	2.82	0.00755
	2.22	2.84	0.0115
	1.11	2.85	0.0173

concentration, *k* increased with a fairly sharp point of inflection at about 27 mM oxalate. The protective effect was evident, however, even in the lowest oxalate concentrations (1.11 mM). In parallel experiments without buffer it was found that the ascorbic acid was slightly less stable in the presence of the buffer; but this was shown only in the lower concentrations of oxalate and was masked by higher concentrations.

#### *The Stability of Ascorbic Acid in Relation to the Initial Concentration in Oxalate*

The effects of varying the concentration of ascorbic acid on its stability in oxalate were determined. The initial concentration of ascorbic acid was varied from 2 to 20 mM and the concentration of oxalate from 1.1 to 111 mM, while the optimum pH was maintained by the use of the citrate-phosphate buffer at one-half strength (Table II, Parts *a*, *b*, and *c*). Over this range of initial concentrations of ascorbic acid the concentration of oxalate required for maximum protection was relatively constant at about 40 mM and was independent, within these limits, of the concentration of ascorbic acid.

This absence of proportionality and the fact that at the low concentration of ascorbic acid the molar ratio of oxalate to it for maximum protection was

1 to 7 (5.68 to 40 mM) shows that a stoichiometric relation is not involved between ascorbic acid and the oxalate required for protection. An increase in the values of  $k$  occurred at the lowest concentration of ascorbic acid.

### *The Effect of Ascorbic Acid on the Stability of Oxalate*

Although it was entirely unlikely that the relatively great stability of ascorbic acid in oxalate would be due to preferential oxidation of the latter, this possibility was checked. It was found, as expected, that at the optimum pH for stability of ascorbic acid, the concentration of oxalate remained unaffected.

### *The Effect of Copper*

Many investigators have shown that copper accelerates the oxidation of ascorbic acid (2, 16, 20, 22, 27, and others). In our experiments, the solutions of ascorbic acid, with various concentrations of copper sulphate, were maintained at pH 2.8 by citrate-phosphate buffer. One series of solutions contained 83 mM oxalate, the other, none. Without oxalate, copper at  $10^{-4} M$  concentration or above greatly accelerated the oxidation of ascorbic acid; but at or below  $10^{-5} M$  had much less effect (Table III). This agrees with the findings of Ghosh (10) that copper added in the order of  $10^{-6}$  mole per liter had little effect on the rate of oxidation.

TABLE III

THE EFFECT OF COPPER SULPHATE AND OXALATE ON THE OXIDATION OF  
ASCORBIC ACID AT 37° C.

Copper concentration	Ascorbic acid after 24 hr. percentage of initial	
	No oxalate	With oxalate, 0.083 M
0	85.5	96.6
$M \times \frac{1}{10^7}$	84.2	96.1
$M \times \frac{1}{10^6}$	82.0	95.9
$M \times \frac{1}{10^5}$	81.0	95.6
$M \times \frac{1}{10^4}$	68.5	95.4
$M \times \frac{1}{10^3}$	59.6	92.1
$M \times \frac{1}{10^2}$	23.6	90.0
$M \times \frac{1}{10}$	5.6	47.2

TABLE IV

THE EFFECTS OF VARIOUS SUBSTANCES ON THE STABILITY OF ASCORBIC ACID (INITIAL CONCENTRATION 5.68 mM) IN SOLUTION AT 37°C.

Substance added		pH	Number of solutions in test	McIlvaine buffer	Effect on the disappearance of ascorbic acid
Name	Concentration, mM				
Borate	16 to 640	3.3	5	—	Increased destruction
Borate	81	2.48 to 6.91	9	+	Increased destruction
Citrate + phosphate	500 and 100 respectively	Below 4	Large number	+	Slightly increased destruction. Usually about 10% less of the initial ascorbic acid remained after 24 hr.
Phthalate	50	2.2 to 6.4	11	—	No effect
Formate	111	2.42 to 4.50	7	—	No effect
Caffeine	1.3 to 0.16	2.92	3	+	No effect
Creatine	20	4.6	8	+	No effect
Creatinine	20	4.6	9	+	Slightly increased stability
Glutathione	1.1	2.6 to 6.9	9	+	Increased stability, see Fig. 3
Thiourea	0.0001 to 100	3.4 to 2.92	8	—	Increased stability, see Table V
Thiourea	10	2.20 to 6.98	10	+	Increased stability, maximal at pH 2.70, $k = 0.0005$
Diethyldithiocarbamate	0.1 to 10	2.91 to 3.34	4	+	Increased stability, see Table VI

The presence of oxalate inhibited the increased oxidation due to copper up to  $10^{-4} M$  concentration. Oxalate also improved the stability in the absence of copper. In solutions with no added copper or oxalate after 24 hr., 85.5% of the initial ascorbic acid remained. When oxalate alone was added, the corresponding value was 96.6%, while the addition of  $10^{-5} M$  copper alone decreased the value to 81%. Thus oxalate had a considerable protective effect in the absence of added copper; while copper added (without oxalate) up to  $10^{-5} M$  had a relatively small destructive effect. The protective effect of oxalate in the solutions without added copper cannot be explained on the possible removal from the reaction of minute traces of the metal that might contaminate the solutions; for the latter quantity in the solutions prepared with triple distilled water was not far above the limit of sensitivity of the

diethylidithiocarbamate test for copper, which is given as 1 part in 100 million, i.e.,  $1.6 \times 10^{-7} M$  (5).

### The Effects of Various Substances

The effects of a number of substances on the stability of ascorbic acid are summarized in Table IV. Borate was investigated since it can react with certain polyhydric alcohols to form complex compounds (14); but it increased the destruction of ascorbic acid in solution, this being more apparent above pH 3. The citrate and phosphate together slightly accelerated the rate of destruction of ascorbic acid but, for some unknown reason, these substances singly had no noticeable effect below pH 4. In confirmation of the observations of Krishnamurthy and Giri (18), it was found that phthalate, formate, caffeine, and creatine were without effect. Creatinine had some protective influence for at pH 4.6 with 20 mM creatinine, and without, the values of  $k$  were 0.0092 and 0.030 respectively. Glutathione (1.1 mM) increased the stability of ascorbic acid (Fig. 3), the maximum occurring at pH 3.5 to 3.9. The glutathione itself was most stable at pH 3 to 4.

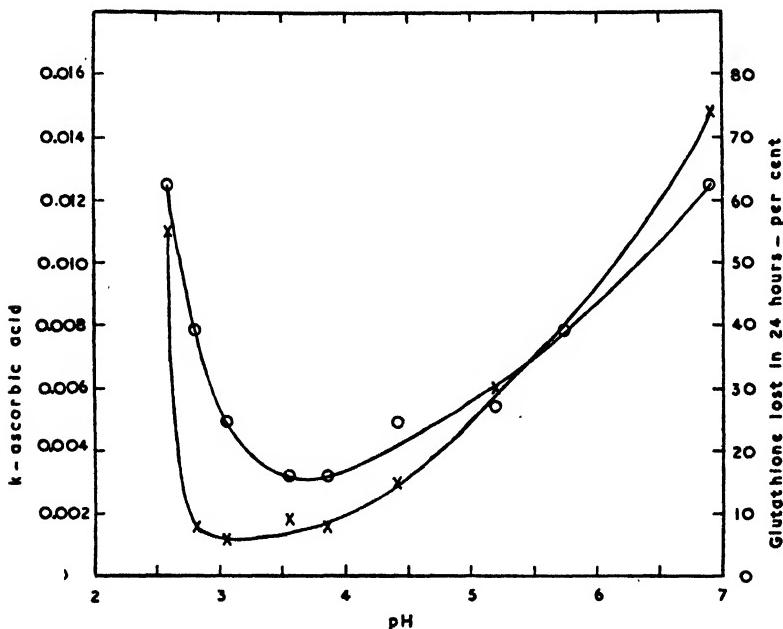


FIG. 3. The effect of pH on the stability of ascorbic acid ○—○; and of glutathione X—X when mixed in solution with citrate-phosphate buffer at 37° C.

Thiourea in low concentrations has been found to protect ascorbic acid in certain foodstuffs from oxidation (Kawerau and Fearon, 15). In the experiment shown in Table V, this substance in 0.1 mM concentration and above increased the stability of ascorbic acid. In another experiment, 10 mM thiourea with citrate-phosphate buffer greatly increased the stability from pH 2.20 to 6.98. The maximum stability ( $k = 0.0005$ ) occurred at pH 2.70.

TABLE V

EFFECT OF THIOUREA ON THE STABILITY OF ASCORBIC ACID. INITIAL CONCENTRATION OF ASCORBIC ACID 5.68 mM; TEMPERATURE 37° C.

Thiourea concentration, mM	pH		<i>k</i>
	Initial	After 48 hr.	
100	3.44	3.40	0.00096
10	3.41	3.38	0.00147
1	3.41	3.36	0.00230
0.1	3.41	3.31	0.00575
0.01	3.41	2.90	0.0218
0.001	3.42	2.90	0.0221
0.0001	3.42	2.92	0.0225
0	3.46	2.92	0.0230

McFarlane (22) found that the addition of sodium diethyldithiocarbamate alone or with  $\alpha,\alpha'$ -dipyridyl protected ascorbic acid from the accelerated oxidation due to copper and iron. In the present study, pH  $3.1 \pm 0.2$  was maintained with citrate-phosphate buffer and the water was triple distilled, as usual. The diethyldithiocarbamate increased the stability of ascorbic acid under these conditions, to the extent that at 10 mM concentration,  $k = 0.00059$  (Table VI). This indicates that the substance can inhibit the uncatalyzed oxidation of ascorbic acid as well as that catalyzed by heavy metals. In these acid solutions some decomposition of the additive occurred, giving rise to a white turbidity and a disagreeable odor of carbon disulphide.

TABLE VI

EFFECT OF SODIUM DIETHYLDITHIOCARBAMATE ON THE STABILITY OF ASCORBIC ACID IN PRESENCE OF CITRATE-PHOSPHATE BUFFER. INITIAL CONCENTRATION OF ASCORBIC ACID 5.68 mM

Sodium diethyl-dithiocarbamate concentration, mM	pH	<i>k</i>
10	3.34	0.00059
1	3.00	0.0023
0.1	2.91	0.0035
0	2.94	0.0063

#### *Spectral Absorption Characteristics of Ascorbic Acid in Oxalate*

The previous experiments indicated that oxalate might protect by depressing the activity of the "enediol" group of ascorbic acid. Since the characteristic absorption peak of ascorbic acid in the ultraviolet can be attributed to this group (11), the effect of oxalate on the spectral absorption of ascorbic acid was determined. A Beckman Spectrophotometer (Cary and Beckman, 7) was used.

In a 5.5 mM solution, oxalate alone absorbed at wave lengths below 230 m $\mu$  and, as increasing concentrations produced greater absorption in the higher wave lengths and thus obscured the peak due to added ascorbic acid, this was the highest concentration of oxalate that could be used. Increasing the pH of the oxalate solutions from 3.33 to 4.51 caused a slight shift of the absorption curve due to this substance to lower wave lengths (Fig. 4).

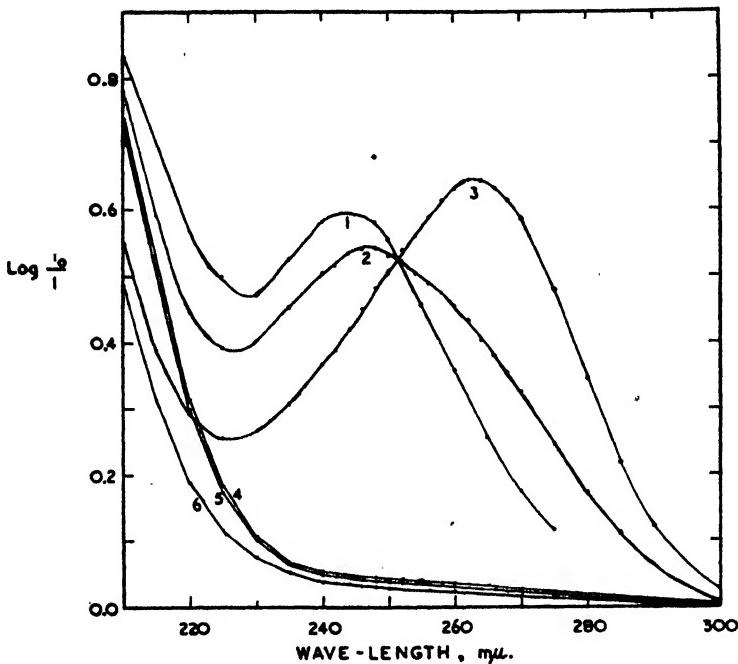


FIG. 4. The spectral absorption by solutions containing 5.68 mM ascorbic acid in 5.5 mM oxalate at pH 3.33, 3.88, and 4.51, Curves 1, 2, and 3 respectively; and by solutions of 5.5 mM oxalate at pH 3.33, 3.88, and 4.51, Curves 4, 5, and 6 respectively.  $l = 1$  cm.

Baird *et al.* (1) found that the wave length at which maximum absorption ( $\lambda_{\text{max.}}$ ) of ultraviolet light by ascorbic acid occurred was influenced by the concentration of hydrochloric acid added. This effect occurred also in our experiments in which other acids were used. In the presence of oxalate the  $\lambda_{\text{max.}}$  for ascorbic acid shifted from 244 to 263 m $\mu$  when the pH was changed from 3.33 to 4.51 (Fig. 4), and similar shifts of  $\lambda_{\text{max.}}$  were observed in formic acid and in orthophosphoric acid.

It was found that the molecular extinction coefficient ( $\epsilon_{\text{max.}}$  as defined by Morton, 23) of ascorbic acid also varied with pH, being minimal at pH 4.0. This occurred in the presence of oxalate, formate, and orthophosphate solutions, the minimal values of  $\log \epsilon_{\text{max.}}$ , ascorbic acid, at pH 4.0 being 3.946 and 3.916 in oxalate and formate respectively. It is interesting that the molecular absorption of ultraviolet light by ascorbic acid is least near the pH of maximum stability; on the other hand, this occurs in the presence of formate

and phosphate, which have no specific protective effect other than through reduction of the pH, as well as in oxalate, which has.

### Discussion

There is an optimum range of pH for the stability of ascorbic acid in solution, which is dependent, at least in part, on the nature of the accompanying substance. In oxalate and in thiourea the optimum range is pH 2.5 to 3.0 while in glutathione it is about pH 3.9. The latter substance is itself most stable from pH 3 to 4.

Oxalate has a strong protective effect on ascorbic acid. In 55 mM oxalate at pH 2.8 and 37° C. the value of  $k$  for the disappearance of ascorbic acid was about 0.0006. The values of  $k$  in 10 mM thiourea at pH 2.7 and in 10 mM diethyldithiocarbamate at pH 3.3 were 0.0005 and 0.0006 respectively. In 1.07 mM glutathione at pH 3.9,  $k = 0.003$ ; but increased concentrations of this substance would most likely increase the protection. Confirming the work of Krishnamurthy and Giri (18) it was found that creatinine had some protective effect but that creatine, formic acid, and phthalic acid had none. The latter result shows that the protective effect is not common to dicarboxylic acids.

There are a number of possible ways by which a substance may inhibit the oxidation of ascorbic acid. The evidence on the protective effect of oxalate may be examined in relation to the possible modes of action of this substance. Borsook, Davenport, Jeffreys, and Warner (4) and Rosenfeld (26) have shown that when oxidation has proceeded to the stage of the formation of oxalate from ascorbic acid, the reaction is irreversible. Thus oxalate cannot be expected to inhibit oxidation by a mass effect.

The evidence indicates that oxalate does not combine chemically with ascorbic acid to form a new compound that is more stable. The concentration of oxalate required for maximum protection was found to be substantially constant and to be independent of the initial concentration of ascorbic acid. The protective effect was present, though submaximal, in low concentrations of oxalate. There was no evidence for a stoichiometric relation between the ascorbic acid and the oxalate required for protection.

An inhibitor of the oxidation of ascorbic acid might act as a hydrogen donor, either reducing dehydroascorbic acid or being oxidized, preferentially, more rapidly. The work of Hopkins and Morgan (12) indicates that glutathione acts in the former way to protect ascorbic acid. Krishnamurthy and Giri (18) failed to obtain reduction of dehydroascorbic acid with oxalic acid, and the observation in these experiments that oxalate at pH 2.8 is stable in the presence of ascorbic acid also relates to this. Apparently, therefore, oxalate does not act as a hydrogen donor to protect ascorbic acid.

It is possible that oxalate may protect by removing the catalytic effect of traces of heavy metals. There is a suggestion of this in the results of the present experiments. It is strongly indicated that oxalate protects ascorbic

acid from oxidation in some other way also, for protection occurred when the effect of heavy metals was negligible. This agrees with the results of Ghosh (10) and Krishnamurthy (17).

From pH 2.4 to 3.0 the solubility of oxalate in mixtures of oxalic acid - sodium oxalate is least; and also the stability of ascorbic acid in lesser concentrations of oxalate is maximum. From the data of Foote and Andrew (9) it seems clear that under these conditions the amounts of the monosodium salt in solution are very large in relation to the amounts of free acid and disodium salt. The correspondence of the maximum stability of ascorbic acid with the highest relative concentration of sodium hydrogen oxalate (or sodium hydrogen oxalate monohydrate) cannot be explained by the effect of pH alone because the maximum stability occurs at a different pH in the presence of glutathione and the effect of pH is apparently not the same in metaphosphoric acid solution (Table I). The optimum pH for stability in thiourea, however, is about the same as in oxalate.

The observation that the addition of ascorbic acid did not alter the solubility of oxalate does not support the possibility that a complex of the two substances may be formed, but to test this properly it would be necessary to use higher concentrations of ascorbic acid.

The ionization constants,  $pK_1$  and  $pK_2$ , of ascorbic acid are given as 4.12 and 11.51 at 22° to 23° C. by Kumler and Daniels (19), so that the optimum effect of oxalate is exerted on the (undissociated) acid form of the vitamin. The hypothesis of Weissberger and LuValle (28) and of Weissberger, LuValle, and Thomas (29) apparently does not cover this pronounced effect.

The "enediol" group of the ascorbic acid molecule is the site of the first step in its oxidation and is responsible for its absorption of ultraviolet light. The pH of the solutions influences not only the wave length at which maximum absorption by ascorbic acid occurs, but also the extent of the absorption, which is minimal at pH 4. Thus the absorption is minimal near the pH of maximum stability. The effect appears to be dependent chiefly on pH, since it occurred in the presence of oxalate, formate, and orthophosphate ions. Of these, oxalate has a specific protective effect against oxidation of the ascorbic acid while the formate and orthophosphate have not. Thus the changes in ultraviolet absorption do not reveal a specific effect of oxalate on the "enediol" group.

The evidence available on the nature of the protection afforded by oxalate appears to eliminate the first four possibilities and leaves the open question: Is ascorbic acid protected by an orientation of oxalate in solution with it so as to cause a depression of the activity of the "enediol" group?

The practical considerations arising from these investigations can be briefly discussed. The ascorbic acid content of citrus fruits is of the order of 2 mM, which is within the range of concentrations investigated (Table II). From the discussion of Jeghers and Murphy (13) it is strongly indicated that the toxicity of oxalate is too high to permit of its addition to foodstuffs. The use of oxalate

as an anticoagulant for blood should, in the presence of acid protein precipitants, have a protective influence on the blood ascorbic acid. The studies have shown, in agreement with those of previous workers, that the stability of ascorbic acid is favored by an acid pH, the absence of copper, and the presence of certain protective substances.

### Acknowledgments

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## A COMPARISON OF THREE METHODS FOR THE ASSAY OF COMMERCIAL INSULIN PREPARATIONS<sup>1</sup>

BY D. M. YOUNG, D. B. W. REID, AND R. G. ROMANS

### Abstract

Each of 14 commercial insulin preparations was assayed for insulin activity using three biological assay methods. In Assay I, 32 rabbits were employed using the "twin cross-over" design. Per cent reductions of blood sugar calculated from blood-sugar levels determined before and one and one-half, three, and five hours after the subcutaneous injection of insulin were used in estimating potency. In Assay II, 16 rabbits were used in a  $4 \times 4$  Latin square design. Blood-sugar levels determined 50 min. after the intravenous injection of insulin were used in estimating potency. In Assay III, 288 mice were employed in a two-level quantal response design. The proportions of animals convulsing in groups of 36 mice after the subcutaneous injection of insulin were used in measuring potency. The agreement between results obtained by the three methods was found to be satisfactory. The average standard error for the estimate of potency was 13% for Assay I; 10% for Assay II; and 9% for Assay III.

The purpose of the present investigation was to compare two rabbit blood-sugar methods and a mouse convulsion method for the determination of insulin activity. Fourteen insulin preparations were assayed by each of the three methods. The estimates of insulin content are presented and some attempt is made to assess the relative efficiency of the different procedures.

### Materials and Methods

Each of the insulin preparations used in the study was assayed for its insulin content by the three assay procedures described below. Eleven of the samples were insulin of crystalline or noncrystalline origin, the products of four North American manufacturers; two were samples of Protamine Zinc insulin; and one was a sample of Globin Insulin with Zinc. Some of the samples were from the open market and some were control samples prepared specifically for animal assay.

#### *Assay Method I. Rabbits Receiving Insulin by Subcutaneous Injection*

Thirty-two rabbits of mixed breed varying in weight from 1.5 to 3.0 kgm. were used in each assay. The rabbits were fed a commercial ration\* and water was available to them at all times. The "twin cross-over" design as used by

<sup>1</sup> Manuscript received August 2, 1949.

Contribution from Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada.

\* Purina Rabbit Chow (Complete ration).

Smith *et al.* (3) was employed. Each rabbit received one dose of Insulin Standard\* and one dose of the unknown under study administered subcutaneously in one ml. of diluting fluid made up to U. S. P. XII (5) specifications. The higher and lower doses of Insulin Standard contained 1.6 and 0.8 International Units respectively. An interval of one week was allowed between the two injections. Samples of blood were drawn from the rabbits at the times and in the manner prescribed by the U. S. P. XII and blood glucose determinations were performed on the individual blood samples by the method of Nelson (2). Per cent reductions of blood sugar, calculated as directed by the U. S. P. XII, were employed in computing the estimates of potency and their standard errors. Reductions in blood sugar of less than 10% were observed in 8% of the animals and these values were arbitrarily excluded from the calculations.

#### *Assay Method II. Rabbits Receiving Insulin by Intravenous Injection*

Sixteen rabbits selected and fed as described under Assay Method I were used in each assay. The experimental design employed was that of Bliss and Marks (1), two doses of Insulin Standard (0.6 and 1.2 I.U. per rabbit) and two doses of the unknown sample being administered to each rabbit, one dose on each of four successive working days. The experimental technique was that of Young and Romans (6). Rabbits were starved for 16 to 18 hr. and injected intravenously with the specified quantities of insulin. Each dose of insulin was dissolved in two ml. of diluting fluid made up to U. S. P. XII specifications. Fifty minutes after injection blood was drawn in the customary manner and blood glucose was determined by the method of Nelson. The blood-sugar values so obtained were used directly in computing the estimates of insulin content and the standard errors of these estimates. Duplicate assays were performed on four of the 14 samples used in the study.

#### *Assay Method III. Mice Receiving Insulin by Subcutaneous Injection*

One hundred and forty-four female mice were used in each assay. The mice varied in weight from 16 to 24 gm. They were fed on a commercial ration\*\* and water was available to them at all times except during the actual performance of an assay. They were starved for varying intervals of time (4, 6, and 16 hr.) before an assay and they were given at least three days' rest between assays. A two-level assay design of the usual type was employed involving 36 animals per dose. Each dose was administered subcutaneously in 0.25 ml. of U. S. P. XII diluting fluid. The higher dose of both standard and unknown was twice the strength of the lower dose. The experimental technique was patterned after that of Trevan and Boock (4). The mice were injected with preparations containing insulin and placed in groups of 12 in glass-covered copper boxes. The boxes were suspended in a water bath maintained at a temperature of 36.5° to 37.5° C. The numbers of mice

\* Insulin Standard S 230, 23.0 International Units per mgm. (Kindly supplied by the Insulin Committee, University of Toronto.)

\*\* Master Fox Breeder Ration (Cube form).

convulsing within 75 min. of the time of injection were recorded. The estimates of insulin content and the standard errors of these estimates were computed by the probit method. Duplicate assays were performed on all of the samples and the results of these were combined.

### Results

In Table I are shown the estimates of insulin content provided by the three methods of assay for each of the 14 insulin preparations used in the study.

TABLE I

ESTIMATES OF INSULIN CONTENT GIVEN BY THREE METHODS OF ASSAY

Insulin sample	Assumed potency I.U./ml.	Estimated insulin content (I.U./ml.)		
		Subcutaneous (32 rabbits)	Intravenous (16 rabbits)	Mouse assay** (288 mice)
1 (Insulin)	40	44.2	39.4	36.2
2 "	20	21.6	18.2	20.3
3 "	20	20.4	23.7	20.0
4 "	20	16.0	17.9	20.8
5 "	40	27.4	27.5 & 27.6*	30.0
6 "	20	21.4	16.4 & 20.4*	24.4
7 "	20	19.5	20.4 & 19.0*	17.3
8 "	20	17.0	20.8	20.0
9 "	20	20.8	17.9	18.2
10 "	20	24.3	19.5	16.1
11 "	20	15.8	23.7	21.2
12 (PZI)	40	38.3	33.4 & 37.4*	36.2
13 (PZI)	40	37.5	44.1	41.6
14 (Globin)	40	39.6	53.0	40.3

NOTE: \* Estimates of potency from two separate assays.

\*\* Combined result of two 144-animal mouse assays.

The table also shows the insulin content assumed for each insulin preparation. Approximate tests of significance, using the standard errors of the individual assays, revealed no real differences among the estimates of potency given by the different methods for any one sample.

The average standard deviation of an individual observation was 7.2% for the subcutaneous rabbit method and 7.9 mgm. % for the intravenous rabbit method. The corresponding average slopes of the response curves were 32.8% and -43.3 mgm. % per log unit. The average slope of the response curves for the mouse method was 4.34 probits per log unit. On the assumption that the assumed potency is not greatly different from the estimated potency, the expected standard error of the estimate of potency would be 13% for a 32-rabbit subcutaneous assay; 10% for a 16-rabbit intravenous assay; and 9% for the combined results of two 144-animal mouse assays. Approximate tests of significance suggested that these standard errors differed significantly from one another.

## Conclusions

Commercial preparations of crystalline or noncrystalline insulin, of Prota-mine Zinc Insulin or of Globin Insulin with Zinc may be as satisfactorily assayed for insulin content by a mouse convulsion method as by either of the rabbit methods employed in this investigation.

Under the conditions described, mouse assays employing a total of 288 mice might be expected to provide more information regarding the potency of a commercial sample of insulin than 16 rabbits used in the intravenous rabbit method, and either of these methods might be expected to provide somewhat more information than 32 rabbits used in the subcutaneous rabbit method.

## Acknowledgment

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## TOXIN PRODUCTION IN AERATED CULTURES OF *CORYNEBACTERIUM DIPHTHERIAE*<sup>1</sup>

By F. F. HOWATT AND G. B. REED

### Abstract

In this paper a comparison is made between the growth and toxin production of *Corynebacterium diphtheriae* in aerated and classical still cultures. It is shown that in well aerated cultures fermentation proceeds more rapidly and toxin appears earlier than in still cultures. However, to obtain maximum results in aerated cultures it is necessary to increase the amount of fermentable substance over that present in the media of still cultures. When cultures are aerated with two to three volumes of air per minute and an extra amount of fermentable carbohydrates is added to the growing cultures at a suitable interval a higher yield of toxin is obtained than in still cultures in similar media. The maximum concentration of toxin, moreover, is reached in the aerated cultures after about 60 hr. of incubation as compared with about 120 hr. in still cultures.

In the extensive literature on the production of toxin by *Corynebacterium diphtheriae*, all authors, with one or two notable exceptions, are in agreement that good yields of toxin are obtained only when the organisms grow as a pellicle on shallow layers of suitable fluid media. This opinion is clearly stated in such early papers as those of Roux and Martin (15) and Aronson (2). Twenty-five years later in a general review of the subject Andrewes *et al.* (1) state: "Many workers believe that toxin is only produced when bacteria form a pellicle . . . . any circumstance which hinders the surface growth of *C. diphtheriae* does not permit the free gaseous exchange which seems so intimately connected with toxin production." Since that time many studies have been made on the ratio of surface exposure to volume of medium, the surface-volume ratio (Pope and Healey (13), Hartley and Hartley (3). Kirkbride *et al.* (7), Pope and Smith (14), Wadsworth and Wheeler (17)). Wheeler and Crowe (18) demonstrated that a reduction in the amount of oxygen in the atmosphere above a surface culture does not seriously affect growth of *C. diphtheriae* but does reduce the yield of toxin.

Siebenmann (16) on the other hand found that the toxin as formed in the pellicle growth tends to concentrate in the surface layer of the culture fluid rather than to diffuse through the mass of the medium. As a result he believes the organisms in the pellicle become partially separated from the nutrients of the medium by the flotation of the toxin and therefore the growth is limited.

With these extensive data on the relationship of toxin production to aeration of the growing *C. diphtheriae*, it is not surprising that Linggood (8) turned to more efficient means of aeration. In a brief paper he has shown that *C. diphtheriae* cultures grown in fluid media aerated by continuous mechanical shaking results in rapid diffuse growth and high yields of toxin. The present

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paper is largely a repetition of Linggood's work with added details, particularly in reference to pH changes and concentration of fermentable substances in the aerated cultures.

### *Medium*

### **Experimental Procedure**

Wadsworth and Wheeler's (17) infusion free proteose peptone medium was used throughout this work. Since the results depend to such an extent upon the constitution of the medium the procedure followed in preparing this standard medium is indicated.

The following solution was made from purified salts.

Sodium chloride.....	5	gm.
Dipotassium phosphate.....	1	gm.
Monopotassium phosphate.....	1	gm.
Magnesium sulphate.....	0.2	gm.
Calcium chloride.....	0.1	gm.
Peptone-Difco proteose.....	20	gm.
Distilled water to.....	1000	ml.

The sodium chloride was dissolved in about one-half the volume of water and the peptone dissolved in this solution. The other salts were dissolved separately in about 50 ml. volumes of water and added to the original solution and the volume made up to 1 liter with distilled water. The pH was adjusted to 7.8 with sodium hydroxide. It was steamed at 100° C. for 30 min., any loss in volume made up with distilled water, and cleared by filtration through soft filter paper.

It was dispensed in 200 ml. amounts in 1 liter pyrex Erlenmeyer flasks. This gives a volume to surface ratio of 0.58, which Wadsworth and Wheeler (17) found to be optimal for their medium. The flasks were loosely plugged with cotton and autoclaved at 115° C. for 30 min.

The following solution was prepared and sterilized by Seitz filtration:

Sodium lactate 70% syrup.....	36	ml.
Maltose.....	15	gm.
Glucose.....	7.5	gm.
Distilled water to.....	100	ml.

Four ml. of this solution was added to each 200 ml. volume of sterile proteose-salt mixture just before inoculation.

This finished medium, assayed for iron by Hill's (6) method showed, in different batches, from 0.00010 to 0.00012 mgm. of iron per ml. Since this amount was fairly close to the 0.00014 mgm. per ml. shown by Pappenheimer and Johnson (11) to be the optimum concentration for diphtheria toxin production in this type of medium and yield of toxin was sufficient for the comparative purposes of this experiment, no adjustment of the iron concentration was made.

### *Inoculum*

One strain of *C. diphtheriae* was used: Park-Williams No. 8 (Toronto) kindly supplied to us by the Connaught Laboratories. During the course of

this work daily cultures were made in the above described medium in 2 ml. amounts in 1/2 in. test tubes incubated at 35° C. in a nearly horizontal position. Sufficient pellicle was formed in one tube in 24 hr. growth to provide inoculum for 1 to 2 doz. 200 ml. flasks.

All inoculations for either still or aerated cultures were made by floating one loop of pellicle from 24 hr. cultures on the surface of the 200 ml. lots of broth in 1 liter flasks.

#### *Still Cultures*

Still cultures were incubated, undisturbed in a well ventilated incubator room at 36° C. Growth was evident in about five hours when small, seemingly isolated, clumps of surface growth could be seen in the vicinity of the original inoculum. After 24 hr. a thin but complete pellicle covered the surface of the medium. This pellicle was not uniform but was dotted with small patches of thicker film. After three days' growth the pellicle was much thickened and wrinkled with festoons of growth descending into the fluid. These festoons appeared to break off subsequently and sink to the bottom of the flasks to produce a heavy sediment. It is apparent that these cultures follow the usual course of toxin producing cultures of *C. diphtheriae* (Pope and Smith (14), Wadsworth and Wheeler (17)).

#### *Aerated Cultures*

Aeration was effected by placing the inoculated flasks in a reciprocating shaking machine operated at 90 excursions, of two inches, per minute. This shaking was sufficient to break the surface of the liquid and produce considerable foam but not enough to wet the cotton plugs. Initially, the flasks to be shaken were placed upon the machine immediately after inoculation. This method, however, resulted in total failure of growth in about 20% of the flasks, despite the use of heavier inoculations. To ensure growth in all flasks, a preliminary still incubation of five hours' duration was allowed for all flasks, following which they were placed on the shaker. This five hours' still incubation, which allows some growth of the organism and spread of the pellicle, was sufficient to eliminate growth failures in the agitated cultures.

With this procedure of initial still incubation the aerated cultures rapidly developed a heavy diffuse growth. The medium becomes definitely cloudy in seven to eight hours and in 24 hr. the growth was heavy and evenly diffuse. In the next two to three days the diffuse growth became increasingly heavy with some threadlike clumping of organisms.

#### *Oxidation-Reduction*

In still cultures of *C. diphtheriae* grown as just described, the potential at polished platinum electrodes submerged in the media gradually falls from a strongly positive to a negative potential of approximately -200 mv. in 24 to 60 hr. (Fig. 1). This is similar to the potential curve for cultures of this species shown by Hewitt (5). When the culture was continually aerated with 5 ml. of air per minute per 200 ml. of medium the negative drift in potential was less

marked to +50 mv. and with 150 ml. air per minute per 200 ml. of medium the negative shift in potential was still less to +150 mv. (Fig. 1). When electrodes were introduced into cultures subjected to continuous shaking, as

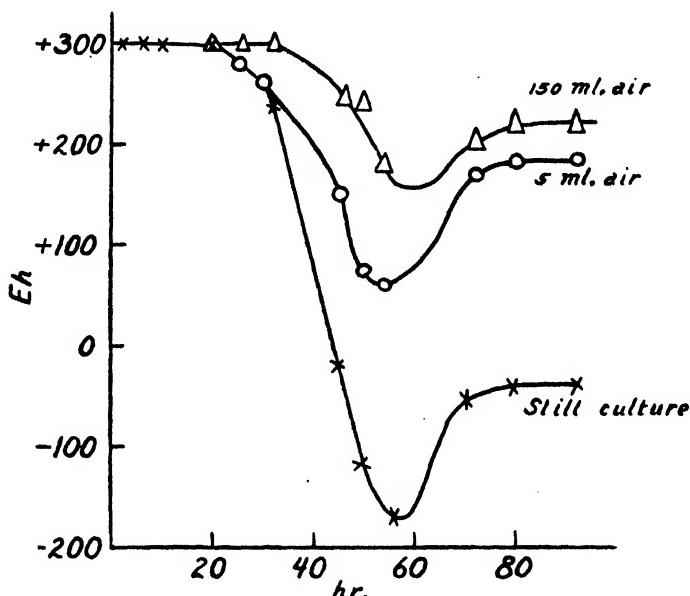


FIG. 1. Graphs showing changes in oxidation-reduction potentials in growing cultures of *C. diphtheriae*, lower curve in still culture; middle curve in aerated culture, 5 ml. air per 200 ml. medium per minute; upper curve 150 ml. air per 200 ml. medium per minute.

described above, it was more difficult to obtain uniform potential readings but the readings fell about midway between those obtained in cultures continually aerated with 5 ml. and 150 ml. air per minute per 200 ml. culture. On this basis it may be estimated that the shaking introduced some 100 ml. of air per minute.

#### pH and Toxin Determinations

At intervals throughout the period of incubation, 5 ml. samples were drawn off from both the shaken and still cultures. In the case of the latter, great care was taken to produce only minimal disturbance of the pellicle and festoons; the flasks were not handled, and the only disturbance was at the point of entry of the pipette into the medium. These samples were centrifuged for 30 min. at about 3500 r.p.m. This was sufficient to throw down the majority of organisms leaving a clear supernatant. This supernatant, containing the toxin, was decanted into a fresh tube and the sediment was discarded.

The pH of the samples was determined with a glass electrode.

#### Toxin Assays

Toxin was assayed by the usual Ramon flocculation test and expressed in Lf units. A partially purified, rapid flocculating standard antitoxin was

obtained from the Connaught Laboratory. This antitoxin contained 2000 units per ml. For routine testing it was diluted to 100 units per ml.

### Still Cultures

### Results

As a preliminary to aeration experiments, toxin production and pH variation in still culture were investigated. Ten numbered flasks were inoculated and placed in the incubator at 35° C. Flasks Nos. 1 to 8 inclusive were sampled at 24-hr. intervals, and the samples were tested for toxin content and pH. Flasks Nos. 9 and 10 were left undisturbed until the end of the experiment to determine whether the entrance of the pipette in sampling caused sufficient disturbance of the pellicle to affect toxin production. The results are shown in Table I and Fig. 2.

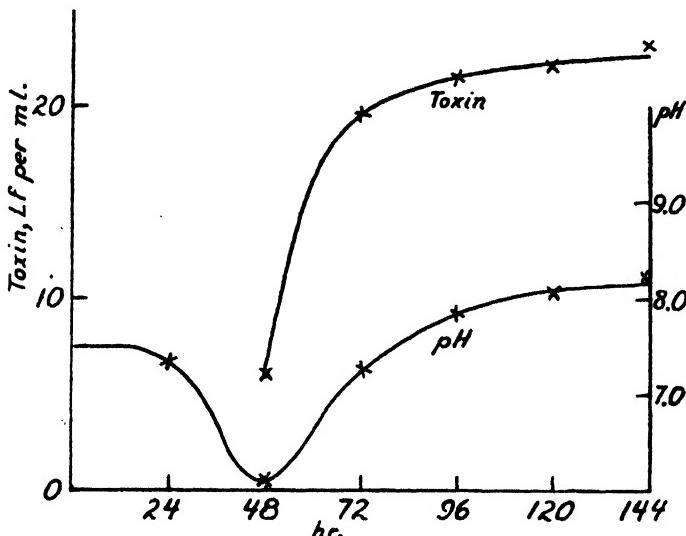


FIG. 2. Graph showing the average pH change and yield of toxin in Lf units per ml. of a group of still cultures of *C. diphtheriae*.

Ordinates represent pH (right), and Lf units of toxin per ml. (left); abscissa time of incubation in hours.

It is apparent that these still cultures followed the usual course of toxin forming *C. diphtheriae* cultures. The pH changes are particularly significant by contrast with aerated cultures discussed in the next section. It is apparent in Table I and Fig. 2 that in these still cultures there is an initial decrease in pH followed by a "reversal" in reaction on the second day and a gradual increase to pH 8.3 on the sixth to seventh day as described by Pope (12) and Andrewes (1). It is well known that the time of "reversal" is related to the surface to volume ratio. With a similar medium and the same surface to volume ratio Wadsworth and Wheeler (17) noted that for high toxin yields the reaction reversal should occur within 72 to 96 hr. and should reach pH 8.0 on the seventh day.

TABLE I  
COMPARISON OF THE pH AND TOXIN PRODUCTION, EXPRESSED AS LF UNITS PER ML., OF 10 SIMILAR *C. diphtheriae* CULTURES

Culture	Time of incubation, hr.																
	24	48	72	96	120	144	168	192	216	168	192	216	168	192	216		
Toxin	pH	Toxin	pH	Toxin	pH	Toxin	pH	Toxin	pH	Toxin	pH	Toxin	pH	Toxin	pH		
1	0	7.4	10	6.1	22	7.4	26	7.8	29	8.0	31	8.1	30	8.3	30	8.3	
2	0	7.3	14	5.9	24	7.4	26	7.8	25	8.0	28	8.3	27	8.4	30	8.3	
3	0	7.4	0	6.2	18	7.2	22	8.0	24	8.1	20	8.3	26	8.2	26	8.3	
4	0	7.2	10	6.2	17	7.3	23	7.7	24	8.0	26	8.3	27	8.4	23	8.4	
5	0	7.3	10	6.1	18	7.2	24	7.8	24	8.1	30	8.1	28	8.3	27	8.0	
6	0	7.5	0	6.2	7.2	7.2	7.8	22	8.1	27	8.2	27	8.3	27	8.3	27	8.0
7	0	7.5	10	6.2	21	7.3	24	7.8	25	8.0	27	8.2	26	8.4	26	8.4	
8	0	7.3	0	6.3	17	7.2	20	7.8	24	8.1	25	8.3	26	8.4	26	8.4	
9																	
10																	
Av.	7.36	6.7	6.15	19.5	7.27	23.5	7.81	24.6	8.05	26.7	8.22	27.3	8.33	27.2	8.35	26.6	8.28

It will be noted that the daily increase in the amount of toxin present in the samples is greatest in the interval between 48 and 72 hr., that is, immediately after the reaction swings towards the alkaline side. In other words, the greatest daily amount of toxin is produced during an interval when the pH is between 6.0 and 7.2. Further production at higher pH is relatively much smaller, although maximum production is reached only when the pH is definitely on the alkaline side.

#### Aerated Cultures

An initial series of 10 flasks of the same lot of medium was inoculated from one 24-hr. culture. After a preliminary five hours' still incubation, five flasks were left undisturbed as still controls and five flasks were placed in the shaker and shaken continuously for six days. At intervals, samples were removed from all flasks for toxin and pH assay. The results are shown in Table II and Fig. 3 where the results on five still cultures are averaged and shown in contrast to the average results in five aerated cultures.

TABLE II

COMPARISON OF THE AVERAGE pH AND TOXIN PRODUCTION, EXPRESSED AS LF UNITS PER ML., OF FIVE STILL CULTURES OF *C. diphtheriae* AND FIVE SIMILAR CULTURES AERATED BY CONTINUOUS SHAKING DURING THE INCUBATION PERIOD

Time, hr.	Still cultures		Aerated cultures	
	pH	Lf/ml.	pH	Lf/ml.
0	7.5	0	7.5	0
5	7.5	0	7.5	0
22	7.4	0	6.7	0
28	7.2	0	7.6	10
34	6.6	0	7.9	20
47	6.1	0	8.3	20
56	6.7	17	8.4	20
72	7.3	20	8.6	19
80	7.5	22	8.7	20
96	7.8	24	8.6	21
116	8.0	25	8.9	20
141	8.2	26	8.8	20

Examination of the curves shows that in agreement with Linggood's (8) results, toxin is formed more rapidly in the aerated than in the still cultures. Reversal of pH occurs earlier in the shaken cultures and in accordance with the findings for still growth, toxin production begins as soon as the reversal reaction occurs. Maximum toxin yield is attained within 36 hr. of inoculation at which time the pH is about 7.9. As incubation proceeds, the pH rises until at 141 hr., it is 8.8 without any further increase in toxin production. This abnormally high pH was noted by Linggood (8), but his report seems to indicate that with his media-(papain digest broth and weak tryptic digest broth) the high pH was attained at the same time (four days) as the maximum toxin yield.

The maximum yield with the Wadsworth and Wheeler medium is lower (20 Lf/ml.) in the aerated culture than in the still culture (26 Lf/ml.). It was considered at first that this might be due to oxidation of the toxin as a

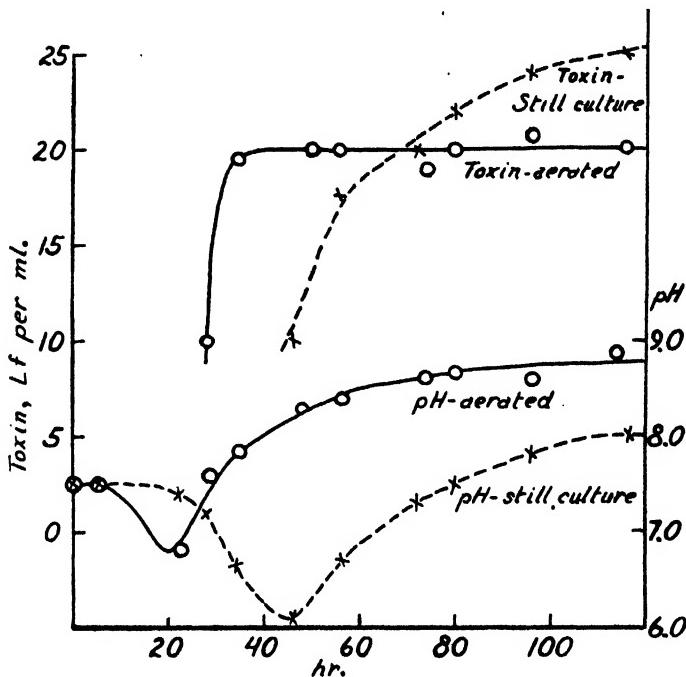


FIG. 3. Graphs showing the average pH change and toxin yield, expressed as Lf units per ml., of still and aerated cultures of *C. diphtheriae*.

Ordinates represent pH (right) and Lf units (left); abscissa, incubation time. Solid line curves represent aerated cultures; dash line curves, still cultures.

result of the increased aeration, but repeat attempts to effect reduction with sodium hydrosulphite according to the method described by Neill (10) failed to increase toxicity.

#### Aerated Cultures with Additional Carbohydrates

The rapid growth and the early pH changes in the aerated cultures suggested, as a second possibility, that the lower production of toxin might be related to early exhaustion of fermentable substances. Locke and Main (9) found that the addition of 0.1% of glucose to growing still cultures, after reaction reversal, resulted in a definite increase in toxin yield. Helson, Stevenson, and Reed (4) similarly increased the yield of *Clostridium botulinum* toxin by adding a medium concentrate to growing cultures.

A series of 24 flasks of the standard Wadsworth and Wheeler medium with the usual concentration of lactate, maltose, and glucose were inoculated from one 24-hr. culture. They were incubated five hours as still cultures then placed in the shaking machine and shaken continuously for six days. The 24 cultures were divided into four lots of six cultures each. To the first three lots a concentrate of lactate, maltose, and glucose was added in amounts equal

to the initial concentration of these substances. To the first series the concentrate was added after 24 hr. incubation of the culture, or approximately at the point of reaction reversal; to the second series after 28 hr., or some four hours after reversal; to the third series after 36 hr. incubation, or 12 hr. after reversal. The fourth series was left as a control with no added fermentable substances. The first three series of cultures had therefore a total concentration of fermentable substances double that contained in the control cultures.

TABLE III

TOXIN PRODUCTION AND pH CHANGES IN FOUR SETS OF AERATED CULTURES OF *C. diphtheriae*:  
I—NO ADDITION TO THE GROWING CULTURE; II—ADDITION, AFTER 24 HR., OF CONCENTRATE  
OF SODIUM LACTATE, MALTOSE, AND DEXTROSE EQUIVALENT TO HALF THE AMOUNT IN  
THE ORIGINAL MEDIUM; III AND IV—SIMILAR ADDITIONS AFTER 28 HR. AND  
AFTER 36 HR. INCUBATION

Time	I. No addition		II. Concentrate added 24 hr.		III. Concentrate added 28 hr.		IV. Concentrate added 36 hr.	
	pH	Toxin	pH	Toxin	pH	Toxin	pH	Toxin
0	7.5	0	7.5	0	7.5	0	7.5	0
5	7.5	0	7.5	0	7.5	0	7.4	0
24	6.8	0	6.8*	0	6.7	0	6.8	0
28	7.6	10	7.0	10	7.5*	10	7.5	10
36	8.0	20	8.0	26	7.8	24	7.9*	20
48	8.3	20	8.1	24	8.2	28	8.2	30
56	8.4		8.2		8.4		8.4	29
72	8.6	19	8.6	23	8.6	28	8.6	30
80	8.7	20	8.4	25	8.6	27	8.9	30
96	8.6	20	8.6	26	8.9	27	8.7	30

\* Indicates time when concentrate of lactate, maltose, and dextrose added.

Results of the several series are shown in Table III and Fig. 4. It will be noted from the table that in the series to which concentrate was added at 24 hr. the average pH at the time was 6.8 or just beyond the point of reversal of the reaction. In the 28- and 36-hr. series the pH had reached 7.5 and 7.9 at the time the concentrate was added, well beyond the reversal point. The addition of this considerable amount of fermentable material, however, had little influence on the pH of the culture. In Fig. 4 the pH curve is drawn through the observed points in the control cultures without added concentrate. The determined points in the cultures to which concentrate was added fall very close to this curve, Fig. 4.

The yield of toxin, in sharp contrast, is conspicuously increased in the cultures to which the concentrates were added. As indicated in Table III and Fig. 4 the aerated cultures to which no concentrate was added gave a maximum yield of 20 Lf units per ml. after some 40 hr. incubation. In the cultures to which the concentrates were added toxin continued to increase up to 50 to

60 hr. and reached a maximum of 30 Lf per ml. It is also apparent from Fig. 4 that the extent of the influence of added concentrate is related to the interval of the addition. When the addition was made at approximately the

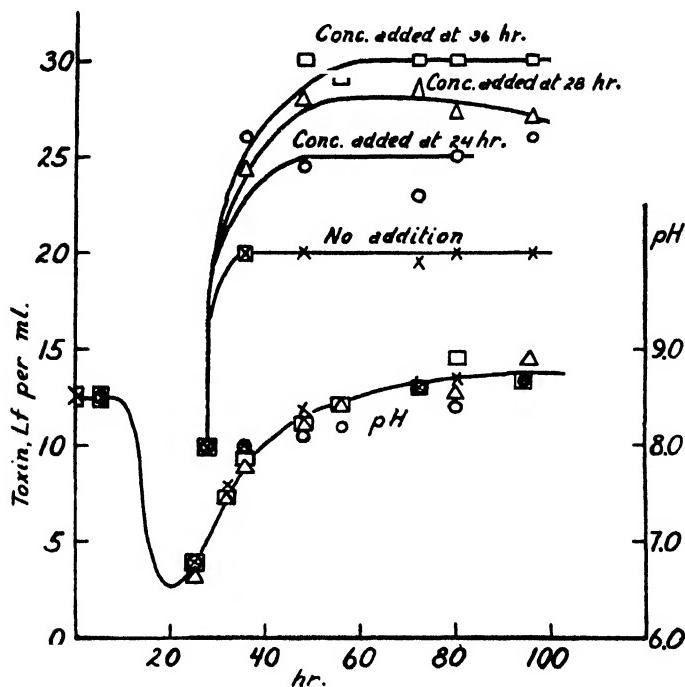


FIG. 4. Graphs showing average pH and toxin yield in four groups of aerated cultures of *C. diphtheriae*. The lower curve indicates pH change in all four groups of cultures. The upper curves show toxin yields, successively, in cultures with no addition of fermentable substances beyond that present in the standard medium and with additions of fermentable substances at 24, 28, and 36 hr. after the beginning of the incubation period.

Ordinates represent pH (right) and Lf units (left); abscissa, incubation time.

point of reaction reversal in the initial culture there was a 25% increase in toxin yield over that in the control culture without addition; when the addition was delayed until some four hours after the reaction reversal there was a 36% increase in the toxin yield and when the addition was delayed to some 12 hr. after reversal there was a 50% increase in toxin yield.

It is apparent from these results that aeration of *C. diphtheriae* cultures markedly alters the metabolism as compared with still cultures. This is evident in the conspicuously different pH curves produced by the two types of cultures, Fig. 3. The difference in metabolism in aerated and still cultures is also evident in the very different oxidation-reduction changes in the two sets of cultures. The most significant difference, however, is the more rapid production of toxin and the higher total yield of toxin, provided an adequate supply of carbohydrates is available, in the aerated cultures as compared with classical still cultures.

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# NEUTRALIZING ANTIBODIES AGAINST MOUSE-ADAPTED LANSING STRAIN OF POLIOMYELITIS VIRUS IN THE SERAS OF ACUTE AND CONVALESCENT CASES AND NORMAL INDIVIDUALS<sup>1</sup>

By E. L. BARTON, N. A. LABZOFFSKY, W. G. ROSS, and L. P. MORRISSEY

## Abstract

The present communication deals with a survey of neutralizing antibodies to mouse-adapted Lansing strain of poliomyelitis virus in the sera of acute, convalescent, and normal individuals during a 1946 epidemic. Two-phase sera were obtained from residents of Ontario and British Columbia and convalescent and normal sera from Quebec. In the sera of 17 out of 35 Ontario patients neutralizing antibodies were demonstrable during the acute stage. In four of these seropositive individuals, there was an increase in the neutralizing titer during convalescence and in three patients there was a notable drop in the titer. The remaining 18 patients were found to be seronegative during both the acute and convalescent stages. Sera from six out of nine British Columbia patients, likewise, contained neutralizing antibodies to the Lansing strain of virus during the acute stage. In four of these the titer remained unchanged during convalescence, in one the titer decreased, and another patient became seronegative. Of the remaining three, two continued to be seronegative and one became seropositive during convalescence. Positive neutralization reactions were obtained with 17 out of 44 convalescent sera from Ontario and 62 out of 146 convalescent sera from Quebec. Sera from 51 children without history of poliomyelitis and 100 adult sera taken at random from specimens submitted for Wassermann tests were obtained from Quebec. Of the children's sera 43%, and of the adults', 48%, contained neutralizing antibodies. The results obtained closely agree with those reported by American workers.

## Introduction

Although a considerable amount of work has been done on the neutralization of mouse-adapted Lansing strain of poliomyelitis virus by human sera, the significance of the antibodies remains obscure.

It is generally agreed that neutralizing antibodies against the Lansing strain of virus in poliomyelitic sera appear irregularly (1, 2, 4, 5, 8). In only a very small percentage of clinically recognized cases of poliomyelitis did antibodies appear during the illness, or increase during convalescence (1, 3, 4, 8). In some cases there was a noticeable decrease in antibody titer or complete disappearance of antibodies during convalescence (1, 4, 8). In most instances, however, the antibodies were either absent or, if present, remained apparently unaltered in titer throughout the course of the disease (1, 2, 4, 5, 8).

Further, it has been shown that a considerable number of newborn children contain in their sera neutralizing antibodies against the Lansing virus, presumably as a result of transplacental transmission (6, 7, 9). A very high percentage of normal children, as well as of normal adults, have in their sera neutralizing antibodies to this strain of poliomyelitis virus.

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Contribution from the Department of Health of Ontario, Division of Laboratories, Toronto, Ontario.

From the observations made by American workers, it appears that contact with the Lansing or antigenically related strain of virus is widespread; although the percentage of seropositive individuals varies considerably in different geographical areas (1). In view of this it was deemed of interest to determine the incidence of this strain of virus in Canada. For this purpose a limited survey of normal and acute and convalescent poliomyelitic sera from residents of Ontario, Quebec, and British Columbia was made. At the time of this investigation outbreaks of poliomyelitis of epidemic proportions were occurring in the two latter provinces, but there were only sporadic cases in Ontario. The results are reported in this communication.

## Material and Methods

### Sera

Two-phase sera were obtained from Ontario and British Columbia and convalescent sera were procured from Ontario and Quebec. Normal sera were obtained in Quebec from normal adults whose blood was being subjected to serological tests for syphilis and from otherwise normal children being admitted to hospital as a result of trauma.

The sera were tested for bacterial sterility upon arrival and were kept stored at 4° C. until used. Our own observations and those of others (8, 10) indicate that the neutralizing titer of poliomyelitis serum is not affected by storage at 4° C. for a considerable period of time.

### Virus

Mouse-adapted Lansing strain of poliomyelitis virus was obtained through the courtesy of the Bureau of Laboratories, Michigan State Department of Health. After passaging the virus three times in mice, several large groups of animals were inoculated. Spinal cords and brain stems from the paralyzed mice were collected and kept frozen in carbon dioxide ice until a sufficient number were available. A 10% suspension of the pooled cords and brain stems was made in 0.85% sodium chloride solution and then centrifuged for five minutes at 2000 r.p.m. The resulting supernatant fluid was distributed in 1 cc. amounts in ampoules that were sealed and stored in a dry-ice cabinet.

Two such pools were used throughout this investigation. Each pool was tested for sterility and titrated in mice before being employed in the tests. The 50% mortality end point of the pooled material was found to be  $10^{-8.5}$ .

For the test, one or two ampoules were thawed, the suspension was again centrifuged for five minutes at 2000 r.p.m., and the supernatant diluted 1:15 with 0.85% sodium chloride, thus yielding a final concentration of virus-infected neural tissue of 1:150. This constant virus dilution was used in the test and when mixed with an equal amount of test or normal serum represented approximately 10 LD<sub>50</sub>.

### Neutralisation Test

Each serum specimen was tested by mixing an equal volume of undiluted serum and the 1:150 constant virus dilution. When comparative titrations

of the sera were made, the following final dilutions of each serum sample: 1 : 2, 1 : 10, 1 : 40, 1 : 160, and 1 : 640 were tested against the above concentration of the virus. In such cases the samples from different bleedings from the same patient were tested simultaneously, allowing the same time of contact between the virus and serum in each mixture. In cases when these titrations revealed a difference in the titer between acute and convalescent serum samples, the particular sera were re-tested using twofold dilutions, the range being determined by the titer found in the first test.

In all cases the mixtures were allowed to stand two hours at 4° C. and one hour at room temperature. Eight mice were inoculated intracerebrally under light anesthesia with each mixture, the dose being 0.03 cc. The sera were tested in groups of 10 to 20, depending on the number of mice available. Known positive and negative serum controls were always included with each test. Inoculated mice were kept under daily observation for paralysis for 21 days. Mice dying during the first 48 hr. after inoculation were not included in the tabulation of the results. If more than one mouse died during the first 48 hr. in any one group of eight, the test was repeated. The test was repeated also if any irregular results occurred in the controls. All mice used were the Swiss strain of albino mice bred in this laboratory, and were 18 to 21 days old.

#### *Interpretation of the Results*

The criterion for a positive serum was survival of four or more mice out of seven or eight mice inoculated, and alive after the first 48 hr. The serum was considered negative if three or less survived. The same criterion was used in the titration of positive sera, the end point being taken as the highest dilution protecting 50% or more of the mice.

As a rule, undiluted sera that gave 50% protection in mice were re-tested and were classified as positive only when the results of re-test were in agreement with the first observations. These criteria, which are similar to those of Brown and Francis (1), were adopted in order that our results might be comparable with those of others.

### **Experimental Data**

#### *Acute and Convalescent Sera*

The results obtained with 35 two-phase sera from patients in Ontario and nine two-phase sera from patients in British Columbia are summarized in Table I.

From Table I it is seen that 17 out of 35 Ontario patients contained neutralizing antibodies in their sera during the acute stage of illness. In four of these there was fourfold or greater increase in the titer during convalescence and in three there was a significant decrease in the neutralizing titer. The remaining 18 patients of the group were found to be seronegative in the acute as well as the convalescent stage.

TABLE I  
SUMMARY OF RESULTS WITH ACUTE AND CONVALESCENT SERA

	Ontario	British Columbia
Total number of patients	35	9
Number positive		
1st bleeding	17	6
2nd bleeding	17	7
3rd bleeding	Not tested	6
Number of paralytic cases in the group	31	8
Number of positives in paralytic group	14	7
Number of nonparalytic cases in the group	4	1
Number of positives in nonparalytic group	3	0
Titer		
Increased	4	1
Decreased	3	2
Unchanged	10	4
Average age		
Seropositive	13.6	9.1
Seronegative	10.2	7.3
For the group	12	8.7
Average time of bleeding		
1st bleeding	5 days	4 days
2nd bleeding	63 days	16 days
3rd bleeding	Not tested	36 days

Six out of nine British Columbia patients were, likewise, found to be positive at the first bleeding. In one of these there was noticeable decrease in the titer when the third bleeding was taken, one patient became seronegative, and in four the titer remained unchanged. Of the remaining three, two continued to be negative but one patient developed antibodies in her blood during convalescence. This patient, a five-year-old girl, was seronegative when tested two days after onset of illness. The second specimen (12 days later) showed, however, antibodies sufficient, in dilution of 1 : 40, to neutralize approximately 10 LD<sub>50</sub> of virus and the third specimen (40 days after onset) in dilution of 1 : 320.

#### *Convalescent Sera*

The results obtained with convalescent sera from Ontario and Quebec are presented in Table II.

Seventeen out of 44 Ontario patients and 62 out of 146 Quebec patients were found to contain neutralizing antibodies in their sera.

In the Ontario group of convalescent sera the incidence of positive reactions was approximately equal among paralytic and nonparalytic cases; in the Quebec group a higher proportion of nonparalytic cases was positive. Whether or not this difference is significant is open to question. However, it must be

TABLE II  
SUMMARY OF RESULTS WITH CONVALESCENT AND NORMAL SERA

	Convalescent sera		Normal sera	
	Ontario	Quebec	Quebec	
			Children	Adults
Total number of sera tested	44	146	51	100
Number of positive sera	17 (39%)	62 (42%)	22 (43%)	48 (48%)
Number of paralytic cases in the group	23	99		
Number of positives in paralytic group	9 (39%)	39 (39%)		
Number of nonparalytic cases in the group	21	47		
Number of positives in nonparalytic group	8 (38%)	23 (50%)		
Average age				
Seropositive	10	9.9	7.3	
Seronegative	8.8	6.6	6.1	
For the group	9.3	8	6.6	Adults
Average time of bleeding	72 days	150 days		

pointed out that the Ontario group was small, the average age was somewhat higher, and the interval between onset and bleeding was shorter.

The distribution of seropositive and seronegative individuals for the two sexes was approximately the same in both the Ontario and Quebec groups of convalescent cases.

#### Normal Sera

*Normal children.*—Out of a group of 51 sera from children in Quebec, normal with respect to history of poliomyelitis, 22, or 43%, were found to be positive. This percentage of seropositives closely approximates that of the convalescent group, although it must be borne in mind that the normal group was considerably smaller than the convalescent (Table II).

*Normal adults.*—For comparison, 100 sera from normal adults from the Province of Quebec were included in this study. Unfortunately the age of the donors was not recorded; it was stated, however, that they were adults. Antibodies against the Lansing strain of virus (Table II) were found in 48 out of 100 sera, or 48%.

#### Discussion

The results presented in this communication on the neutralization of mouse-adapted Lansing strain of poliomyelitis virus by acute, convalescent, and normal human sera agree in general with the findings reported by American

workers. The significant point of this investigation is that further proof is given of the widespread distribution of the Lansing or antigenically related strains of poliomyelitis virus in different geographical areas. The number of sera from Ontario and British Columbia, while large enough to indicate that this virus exists in those areas, is not large enough to provide an estimate of its prevalence.

Findings with the two-phase sera confirm the observations made by others (1, 3, 4, 5, 6, 8) that a considerable percentage of patients contain in their blood stream antibodies against the Lansing strain of virus during the acute stage. The antibody titer in these cases, with a few exceptions, remained the same throughout the illness. In several patients there was noted a considerable decrease in the titer and in one instance a patient who was seropositive during the acute stage later became seronegative. The last phenomenon has been observed by others (1, 8) and offers interesting speculation. Current investigations into the "interference phenomenon" may throw some light on the problem. The case of those patients whose serum contained antibodies in the acute stage of the disease and showed an increased titer during convalescence might be explained in one of two ways: first, that those patients had had a latent infection, which would have given rise to antibodies and later had developed into frank illness; second, that those patients had had a previous infection that had left residual antibodies, but not sufficient protection to prevent reinfection with the same, or an antigenically related, strain of virus. The possibility of such a phenomenon has been suggested by Brown and Francis (1).

The case of the initially seronegative patient who became positive during convalescence can most reasonably be interpreted as a primary infection with Lansing or related virus.

The average age of the positive groups was found to be always higher than that of the negative groups, a fact that has been established by others (1, 7, 8, 9). The proportion of positive sera was approximately the same in convalescent children as in normal children and adults.

It would appear that the Lansing strain of virus alone was not the cause of these outbreaks. The high percentage of seropositive individuals, on the other hand, suggests that there had been a recent and rather widespread contact with the Lansing or antigenically related strain in these three provinces of Canada.

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# THE RELATIONSHIP OF DIETARY FACTORS TO RAT SERUM ALKALINE PHOSPHATASE

## 1. THE EFFECT OF FAT, METHIONINE, AND CYSTINE<sup>1</sup>

BY JULES TUBA AND RIDLEY K. SHAW

### Abstract

In synthetic diets fed to weanling rats, methionine and fat must be present in a definite ratio in order to maintain a serum alkaline phosphatase activity equal to that obtained on a standard laboratory diet of animal checkers. This ratio is approximately 1 : 25 by weight for a diet containing 8.5% fat. Increased fat enhances, while increased methionine lowers, the serum phosphatase activity. Although in some experiments methionine was fed in concentrations sufficient to lower phosphatase activity to what has been considered definitely subnormal values, growth was good and the general condition of the animals was excellent. However, beyond certain concentrations of the amino acid, food consumption decreased and weight losses occurred. Cystine had no effect in opposing the action of methionine on serum alkaline phosphatase.

### Introduction

There is extensive evidence in the literature that the concentration of alkaline phosphatase in rat serum may be rapidly and significantly altered by a number of dietary factors or the nutritional state of the animal. Weil and Russel (13) found that starvation decreased plasma phosphatase activity and that these lowered levels were elevated following the ingestion of certain unsaturated fatty acids, while saturated fatty acids, proteins, and carbohydrates had no effect. Hough *et al.* (6, 7) observed a pronounced rise in the serum phosphatase concentration of dogs that received a diet high in fat and low in protein. Their work indicated that the level of the enzyme varied directly with the concentration in the diet of labile methyl groups in the form of protein, methionine, or choline. They found that cystine increased the serum alkaline phosphatase activity and attribute to cystine an antilipotropic effect, as do Salmon (8) and Treadwell (10).\*

Considerable work has already been reported from this laboratory concerning the effect on serum alkaline phosphatase of a variety of experimental conditions. Tuba, Baker, and Cantor (11) found a pronounced diurnal variation in the level of the enzyme, associated with the time of feeding, which necessitated bleeding the animals at 8 a.m. in order to standardize the effect of this factor. They noted further that the enzyme concentration varied directly with the total daily consumption of the stock laboratory diet. It is evident from their results that the influence of sex, castration, and sex hormones upon the enzyme activity can be directly correlated with alteration in the quantity of food ingested daily. Cantor, Wight, and Tuba (2) presented further evidence

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\* This confirms the original observation of A. W. Beeston and H. J. Channon. *Biochem. J.* 30 : 280. 1936.

relating food intake to serum phosphatase activity, as well as proof that it is the fat content of the diet rather than the caloric intake that determines the level of the enzyme. Tuba, Cantor, and Richards (12) fed to weanling rats a diet consisting chiefly of barley with a low protein content, which elevated the serum alkaline phosphatase values about 75% above that obtained on a stock laboratory diet. The addition of methionine to the low protein barley diet resulted in very nearly normal concentrations of the enzyme.

The work reported here is an attempt to elucidate the relationships between serum alkaline phosphatase and dietary fat, methionine, and cystine.

### Experimental

Immature, male albino rats were caged individually and given food and water ad libitum. Each diet was fed to a group of 10 animals if possible for a period usually of six weeks. The rate of growth and food consumption of each animal was noted.

Bleeding for estimation of serum alkaline phosphatase concentration was from the tail. Serum was stored at 5° C., when necessary, with unaltered phosphatase activity but enzyme levels were determined within two to three days after the blood was collected. Enzyme values were obtained for each group at intervals during the feeding period. Serum phosphatase was determined by the micromethod of Shinowara, Jones, and Reinhart (9), as modified by Gould and Schwachman (5). The unit of phosphatase activity is defined by Shinowara as "equivalent to one milligram of phosphorus liberated as phosphate ion during one hour of incubation at 37° C., with a substrate containing sodium  $\beta$ -glycerophosphate, hydrolysis not exceeding 10% of the substrate and optimum pH of the reaction mixture for the alkaline enzyme at 9.3  $\pm$  0.15."

The diets all contained 4% McCollum's Salt Mixture, 2% cod liver oil, 0.1% choline chloride, and adequate supplements of thiamine, pyridoxine, pantothenic acid, nicotinic acid, and riboflavin (12). The remaining 94% was made up with sucrose, Crisco, and vitamin-free casein in the proportions indicated below for each diet. Protein was maintained at levels no higher than 10% because experiments to be published in a subsequent paper indicate that 10% protein would permit a steady though subnormal rate of growth, and at the same time this low value of protein would keep at a minimum the lipotropic action of this dietary factor.

### Results

#### THE EFFECT OF VARYING THE DIETARY FAT CONCENTRATION

Weanling male rats, 21 days old, were placed on diets containing 10% protein, vitamin and salt supplements, fat as indicated in Table I, and sucrose in amounts required to complete each diet. Groups of 10 animals were maintained on each diet for six weeks and the average daily consumption, as well

as the average alkaline phosphatase values for each group, are given at two-week intervals. The standard deviations are indicated for the terminal phosphatase values. The growth rates were almost identical and are not included.

TABLE I

THE EFFECT OF DIETARY FAT CONCENTRATION ON AVERAGE SERUM ALKALINE PHOSPHATASE LEVELS (UNITS PER 100 ML.) (P) AND AVERAGE DAILY FOOD CONSUMPTION (GM. PER DAY) (C) OF WEANLING RATS

Diet No.	Fat in diet, %	2 weeks		4 weeks		6 weeks	
		P	C	P	C	P	C
1	25.0	252	4.7	244	5.3	237 $\pm$ 41*	5.9
2	8.5	98	5.9	103	6.2	99 $\pm$ 12	7.0
3	5.0	74	5.6	95	9.1	82 $\pm$ 13	8.9

\* Standard deviations are indicated for phosphatase values at end of experiment.

The results in the above table show clearly that increased fat concentration enhanced the serum alkaline phosphatase activity, although at the same time the daily consumption of food tended to decrease. This is in complete agreement with previous work in this laboratory, although Weil and Russel were able to obtain such parallelism only up to a dietary concentration of 8% lard.

#### THE EFFECT OF METHIONINE

Because both methionine and fat have been shown to influence serum alkaline phosphatase levels, it seemed very likely that the ratio of these two in the diet (M : F) might be of critical importance in determining the concentration of the enzyme. In the work reported previously from this laboratory on the effect of methionine (12) the ratio of the total amino acid (in protein and supplement) to fat was 1 : 70 and this produced an abnormally high enzyme activity. Diet 1 above was designed to have a ratio of M : F\* = 1 : 70, and again very high phosphatase values resulted. In Diet 2 the M : F ratio was 1 : 25 and this effected a level of enzyme activity approximately equal to that obtained with the stock laboratory diet of fox checkers. The M : F ratio in Diet 3 was 1 : 14 and reduced the enzyme activity to subnormal values.

The following two diets were designed to further clarify this apparent relationship between these dietary factors and the serum enzyme. The rates of growth were similar to those for the three diets described above.

#### Diet 4

This contained 5% fat and a supplement of 0.35% DL-methionine, which, taking into consideration the methionine content of the 10% casein, resulted in a ratio for M : F of 1 : 7. A group of eight weanling rats was fed this diet

\* The methionine content of casein was based on values given in Block and Bolling (1).

for the usual six-week period. The increased M : F ratio produced by the fifth week a very pronounced lowering of the enzyme activity, which remained at this level until the termination of the experiment. In Table II this is compared with Diet 2 (M : F = 1 : 25), which also contained 5% fat and produced "normal" enzyme activity. The consumption was slightly greater

TABLE II

THE EFFECT OF INCREASING THE METHIONINE TO FAT RATIO (M : F) ON AVERAGE SERUM ALKALINE PHOSPHATASE (UNITS PER 100 ML.) (P) AND AVERAGE FOOD CONSUMPTION (GM. PER DAY) (C)

Diet No.	M : F ratio	2 weeks		4 weeks		5 weeks		6 weeks	
		P	C	P	C	P	C	P	C
2	1 : 25	98	5.9	103	6.2	92	6.4	99 ± 12*	7.0
4	1 : 7	98	7.7	90	8.7	50	7.7	45 ± 10	7.7

\* Standard deviations are indicated for phosphatase values at end of experiment.

with Diet 4 than with Diet 2, and this ordinarily should have increased the phosphatase activity. The rate of growth of the animals was normal and they appeared to be in good health although such low phosphatase levels on the standard diet of fox checkers would have indicated some abnormal condition.

#### Diet Five

A group of 10 weanling rats was fed a diet containing 25% fat and 10% protein (M : F = 1 : 70) for three weeks, and then 1.45% methionine was added as a supplement (M : F = 1 : 14). A very high enzyme concentration produced by three weeks on the high-fat, low-methionine diet was decreased 45% in 14 days by the methionine supplement, as indicated in Table III. However, the high fat concentration counteracted the methionine effect to such an extent that enzyme activity was not restored to normal, much less to the

TABLE III

THE EFFECT OF A METHIONINE TO FAT RATIO OF 1 : 14 IN RELATION TO VARIATION OF THE FAT CONTENT OF THE DIET ON AVERAGE SERUM ALKALINE PHOSPHATASE (UNITS PER 100 ML.) (P) AND AVERAGE FOOD CONSUMPTION (GM. PER DAY) (C)

Diet No.	Fat, %	0 days		2 days		7 days		14 days	
		P	C	P	C	P	C	P	C
3	5	—	—	—	—	—	—	74 ± 14*	5.6
5	25	296	6.0	195	4.4	171	4.6	167 ± 35	5.0

\* Standard deviations are indicated for phosphatase values at end of experiment.

subnormal values obtained with Diet 3, which also had  $M : F = 1 : 14$ , but contained only 5% fat. A diet containing  $M : F = 1.7$  was fed to these same animals, but this methionine supplement of 3.6% resulted in a loss of weight. This experiment was discontinued because the loss in weight, which always results in decreased serum alkaline phosphatase activity, would inevitably have masked the effect of the methionine itself.

### THE EFFECT OF CYSTINE

The methionine-opposing action of cystine is said to be most marked on a high-fat, low-protein diet. Treadwell (10) has indicated that such a ration will produce fatty livers in rats. It was shown with Diet 5 above that weanling rats subjected to this regime would in three weeks manifest serum alkaline phosphatase levels as much as 200% above normal. Methionine supplements introduced at the end of the three-week lipogenic period had a potent influence in diminishing phosphatase activity. It seemed probable that counter effects of cystine and methionine on the enzyme might also be best demonstrated when concentrations in serum were at high levels.

#### *Diets 6 and 7*

Twenty weanling rats were fed a basal diet of 10% casein and 25% fat for three weeks. Growth curves were the same as previously obtained with this diet. A supplement of 1.8% L-cystine was then fed to a group of 10 of the animals (Diet 6) and the remaining 10 received supplements of 1.8% L-cystine and 1.45% DL-methionine (Diet 7). Consumption and serum phosphatase activities are recorded in Table IV at the termination of the three-week period on the basal diet and at intervals for three weeks while the amino acids were being fed. The supplementary cystine did not in this three-week interval

TABLE IV

THE EFFECT OF SUPPLEMENTARY CYSTINE AND METHIONINE ON AVERAGE SERUM ALKALINE PHOSPHATASE (UNITS PER 100 ML.) (P) AND AVERAGE FOOD CONSUMPTION (GM. PER DAY) (C) OF RATS PREVIOUSLY FED A DIET CONTAINING 25% FAT AND 10% PROTEIN

Diet No.	Supplement	0 days		2 days		1 week		2 weeks		3 weeks	
		P	C	P	C	P	C	P	C	P	C
6	1.8% cystine	235	6.0	254	7.4	235	7.0	206	7.6	198 ± 24*	9.5
7	1.8% cystine, 1.45% methionine	250	7.4	167	4.6	174	6.6	161	6.6	165 ± 27	7.4

\* Standard deviations are indicated for phosphatase values at end of experiment.

produce an enhancement of enzyme activity, as reported by Hough *et al.* (7) on a high-fat, low-protein, low-choline diet. In fact, a slight decrease occurred in phosphatase levels below the values obtained with Diet 1. Although not shown in Table IV, Diet 6 was fed for five weeks by which time the animals

lost weight quickly, enzyme activity decreased, and the animals began to die. Curtis and Newburgh (3, 4) showed that if cystine were fed in amounts exceeding growth requirements death would eventually result. Diet 7 resulted in phosphatase levels identical with those obtained with Diet 5. There was with this diet no demonstrable opposition between cystine and methionine with regard to serum alkaline phosphatase activity.

A choline-free diet, otherwise identical with Diet 6, was used in a further experiment similar to the above two. The addition of cystine to this diet resulted in an improved nutritive state: food consumption and growth rate increased, and phosphatase levels increased but not beyond those obtained with Diet 6. Paired-feeding experiments with the choline-free diet also failed to show any cystine effect.

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## STEROID EXCRETION OF INFANTS<sup>1</sup>

By W. F. PERRY AND H. CHOCHINOV

### Abstract

The urinary excretion of corticosteroids and ketosteroids was measured in infants during the first week of life and at four and 18 months of age. It was found that corticosteroid excretion increased with age while the excretion of ketosteroids declined from birth, but was again at a similar level to that of one- to three-day-old infants in the 18-months-old child. The excretion of androgenic substances did not parallel the excretion of ketosteroids, these substances being present in minute amounts at birth and rising gradually with increasing age. It is suggested that the involution of the fetal adrenal cortex is associated with a declining production of nonandrogenic ketosteroids.

### Introduction

The adrenal cortex of infants is of interest in that it is relatively larger than that of adults and, in addition to the definitive or adult type of cortical cells, there is present a wide zone of cells variously known as the fetal cortex, the "androgenic" zone or the "X" zone. This zone undergoes involution rapidly during the first two weeks postpartum and then more slowly until, by the end of the first year of life, the process is complete. As the significance of this change is obscure it was decided to determine whether it was associated with alterations in the excretion of ketosteroids and corticosteroids, i.e., substances of adrenal cortical origin.

### Subjects and Methods

The subjects were essentially healthy and consisted of 24 male infants between one and seven days old, a two months premature female infant, and two male infants aged four and 18 months. It was found impossible to make accurate and complete 24 hr. collections from these subjects, so it was decided to measure excretions as amounts per liter of urine rather than as amounts per 24 hr. In order to obtain sufficient quantities of urine for assay of androgenic activity the subjects between the ages of one and seven days were divided into two groups made up respectively of 15 infants between the ages of one and three days and nine infants between the ages of four and seven days. Four of the infants were in both groups at the appropriate time after birth. The urine of the members of each group was pooled, no one individual contributing more than 20% of each pool. No preservative was used but the varying amounts of urine collected each day from each child or group of children was immediately frozen and kept so until the various determinations were performed. Approximately 1800 ml. of urine was collected from each group of children; in the case of the premature and the two older infants a similar amount was collected from each child.

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*Contribution from the Department of Physiology and Medical Research of the University of Manitoba, and from the Winnipeg General Hospital, Winnipeg, Canada.*

Ketosteroids were determined by means of their characteristic reaction with *m*-dinitrobenzene in an alkaline medium to form a red pigment. The corticosteroids were determined using principles of the methods of Heard and Sobel (3) and Talbot *et al.* (7). The procedure (6) is essentially an estimation of the reducing power of the neutral lipid soluble substances of urine and for normal adults gives values between 0.5 and 0.9 mgm. per 24 hr. The test for androgenic activity was carried out after the method of Frank *et al.* (2). This test is based on the growth of chick combs following daily topical application, for seven days, of the test substance dissolved in alcohol, androsterone being used as a reference standard. The urine extracts were prepared by acid hydrolysis of 1500 ml. of urine followed by extraction with carbon tetrachloride. The carbon tetrachloride was washed with alkali and water and then evaporated to dryness. The dry residue was taken up in 2 ml. of ethanol and 0.05 ml. was applied daily to each chick. Four to five chicks were used for each unknown and for each of the doses of the standard.

### Results

Although accurate daily outputs of urine were not obtained, one may approximate the daily steroid output by taking the average daily urine volumes to be 70 ml. for the one- to three-day-old infants, 140 ml. for the four- to seven-day-olds, 300 ml. for the four-months-old infant, and 500 ml. for the 18 months child. In Table I the observed concentrations of steroids and the calculated outputs are recorded. It will be seen that the excretion of corti-

TABLE I  
STEROID CONTENT OF INFANTS' URINE

Subjects	Corticosteroids		Ketosteroids		Androgenic activity as androsterone		
	Mgm./liter	Mgm./24 hr. (calculated)	Mgm./liter	Mgm./24 hr. (calculated)	μgm./liter	μgm./24 hr. (calculated)	% total ketosteroids
Premature	0.47	—	7.0	—	0	—	0
1 to 3 days	1.01	0.07	16.0	1.1	18	1	0.1
4 to 7 days	0.43	0.06	6.5	0.9	18	2	0.3
4 months	0.55	0.17	1.6	0.5	32	10	2.0
18 months	0.56	0.28	2.7	1.3	120	60	5.0

costeroids increased after the first week while the ketosteroid excretion declined from birth to four months and increased again in the case of the oldest infant. The androgenic activity did not parallel the ketosteroid excretion but showed a continuous increase with age. As well as an absolute increase in androgenic excretion the percentage of the total ketosteroids with androgenic activity also increased with age.

### Discussion

Ketosteroids and corticosteroids were excreted in higher concentrations by infants one- to three-days-old than by those who were older. This suggests a

postpartum excretion of substances of maternal origin but the very low androgenic potency of the ketosteroids excreted at that time does not support this view. It is more likely that the change in concentration is a reflection of the change in water excretion.

The concentration of corticosteroids in infants' urine is similar to that of adults, though the daily excretion is much lower. Matson and Longwell (4) noted that the excretion of lipid soluble reducing substances by infants was much lower than by adults and considered that the amounts excreted had no correlation with the age or prematurity of the child. However, if the daily excretion were calculated on a body weight basis it appeared that the excretion of these substances was greater in infants than in adults and they suggested that this greater excretion might be due to loss of stored materials occasioned by the involution of the cortex. The progressive increase with age found in the present series does not support this view.

Day (1) found the ketosteroi d excretion of infants between the ages of one and five days to be from 0.1 to 3.4 mgm., averaging 0.9 mgm., similar values to those reported here. More recently Venning (8) has explored in detail the changes in ketosteroi d excretion during the first nine days of life. In three infants she has found excretions between 1.5 and 1.8 mgm. during the first day with a progressive fall each day thereafter. This, too, is in conformity with the present findings.

Only a small proportion of the ketosteroi d material of the infants was found to have androgenic activity but this proportion increased with age. This is contrary to a recent report (5) that there are no androgens present in the urine of children until the age of eight years. However, Wood and Gray (9) on the basis of the difference in values obtained by estimating ketosteroi ds by the Zimmerman and antimony trichloride reagents, the difference representing the amount of dehydroisoandrosterone, conclude that children between the ages of two and eight years excrete relatively more of this poorly androgenic steroid than do adults and further that the younger children excrete relatively more than do older children. Dehydroisoandrosterone is considered to be about 1/10th as active as androsterone. If all the ketosteroi d material in the infants' urine were this substance the biological activity in the case of the one- to three-day infants, for example, would be equivalent to 1.6 mgm per liter, whereas it was only 18  $\mu$ gm. per liter. In other words the majority of the ketosteroi ds in infants' urine are androgenically inert, though the proportion increases with age.

From the evidence presented here it is suggested that the involution of the "X" or "androgenic" zone of the infant adrenal cortex is accompanied by a declining production of nonandrogenic ketosteroi ds. The growth of the adult type of cortex as the infant ages is associated with gradual increase in output of corticosteroids and of ketosteroi ds with androgenic activity.

### Acknowledgment

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# A NOTE ON THE EFFECT OF AN INTRAVENOUS INJECTION OF BILE SALT ON THE PLASMA PHOSPHATASES OF THE RABBIT<sup>1</sup>

By W. F. HAIGHT

## Abstract

Since it has been established (*a*) that the alkaline phosphatase activity of the plasma is increased in obstructive jaundice, (*b*) that the white cells are rich in alkaline phosphatase, (*c*) that this alkaline phosphatase is liberated from the cells in the presence of bile salts, and (*d*) that bile salts accumulate in the blood when the biliary tract is obstructed, there remained the possibility that the increase in plasma alkaline phosphatase observed after obstruction to the biliary tract might be the result of the liberation of alkaline phosphatase from the white cells by the retained bile salt. However the injection of bile salt into the blood stream of rabbits caused an increase in the acid phosphatase activity of the plasma with no significant change in the concentration of alkaline phosphatase. Since there is no increase in the acid phosphatase of the plasma in obstructive jaundice, these experiments provide no evidence for the theory that the increase in plasma alkaline phosphatase that follows biliary obstruction is the result of the retention of bile salt.

## Introduction

Previous work in this laboratory has shown that white cells are rich in alkaline phosphatase (9) and that this alkaline phosphatase is liberated from the white cells by the *in vitro* action of surface-active substances, such as bile salt, saponin, or alkyl sulphate (22). It has frequently been suggested that, if this mechanism were active *in vivo*, it could explain the high plasma alkaline phosphatase values observed after obstruction to the biliary tract, since in this condition surface-active bile salt is retained in the blood. A high concentration of bile might liberate alkaline phosphatase from the white cells of the peripheral blood or from those of other organs rich in white cells, e.g. spleen or bone marrow. Such an explanation of the increase in plasma alkaline phosphatase observed in obstructive jaundice would amount to a revival, in a modified form, of the 'activator' theory of Thannhauser, Reishel, Gratton, and Maddock (27).

This suggestion has been put to the direct test by injecting bile salts into the blood stream of rabbits and observing the changes in both the acid (pH 4.9) and the alkaline (pH 9.9) phosphatase of the plasma.

## Methods

Either sodium desoxycholate or sodium taurocholate, in the quantities and concentrations shown in Tables I and II, was injected into the marginal ear vein of a rabbit. Thirty minutes later blood was withdrawn from the vein of the opposite ear for the determination of the plasma phosphatases. The acid phosphatase was determined by the method of Watkinson, Delory, King, and Haddow (29) and the alkaline phosphatase determined by the method of King, Haslewood, Delory, and Beall (15).

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Contribution from the Department of Biochemistry, University of Western Ontario, London, Ont. The research was supported by a grant from the National Research Council of Canada.

### Results

Table I shows that 30 min. after the injection of 125 mgm. of sodium desoxycholate there was a great (565%) increase in the activity of the acid phosphatase of the plasma with only a negligible (19%) increase in the activity

TABLE I

**THE EFFECT OF INTRAVENOUS SODIUM DESOXYCHOLATE ON THE SERUM ACID AND ALKALINE PHOSPHATASE OF THE RABBIT**

Volume injected, ml.	Conc. of sodium desoxy-cholate, %	Total quantity injected, mgm.	Acid phosphatase activity, King-Armstrong units/100 ml.			Alkaline phosphatase activity, King-Armstrong units/100 ml.		
			Before	30 min. after	Per-cent-age increase	Before	30 min. after	Per-cent-age increase
5	2.5	125	7.71	51.30	565	3.31	3.93	19
4	2.5	100	16.50	53.10	222	4.84	5.66	17
3	2.5	75	8.88	11.91	34	2.87	3.51	22
5	0.5	25	16.90	15.45	-3	4.36	4.67	7

of the plasma alkaline phosphatase. Lesser quantities of sodium desoxycholate (100 mgm. and 75 mgm.) produced a similar, though smaller, increase in acid phosphatase activity. When the quantity injected was reduced to 25 mgm., there was no significant increase in the activity of either phosphatase.

Table II shows that sodium taurocholate had an action similar to that of sodium desoxycholate, except that the quantity necessary to produce the same effect was greater. Injection of 1000 mgm. of sodium taurocholate produced

TABLE II

**THE EFFECT OF INTRAVENOUS SODIUM TAUROCHOLATE ON THE SERUM ACID AND ALKALINE PHOSPHATASE OF THE RABBIT**

Volume injected, ml.	Conc. of sodium tauro-cholate, %	Total quantity injected, mgm.	Acid phosphatase activity, King-Armstrong units/100 ml.			Alkaline phosphatase activity, King-Armstrong units/100 ml.		
			Before	30 min. after	Per-cent-age increase	Before	30 min. after	Per-cent-age increase
10	10	1000	3.27	57.30	1655	5.12	8.16	59
5	10	500	7.02	30.00	327	2.19	2.27	4
5	10	500	12.90	33.60	160	5.69	6.62	16
1	10	100	9.75	10.38	6	4.79	4.56	5

a great (1655%) increase in the acid phosphatase activity of the plasma and a very much smaller (60%) increase in the activity of the alkaline phosphatase. The injection of 500 mgm. sodium taurocholate produced a considerable increase in the activity of the acid phosphatase, but no great change

in activity of the alkaline phosphatase. When the amount injected was reduced to 100 mgm., the sodium taurocholate was without effect.

In the experiments presented in Tables I and II the phosphatase activity was measured 30 min. after the injection of the bile salt. Fig. 1 shows that the plasma acid phosphatase activity slowly decreased from this time and that 24 hr. after the injection both the acid and the alkaline phosphatase activity had fallen to within the normal range.

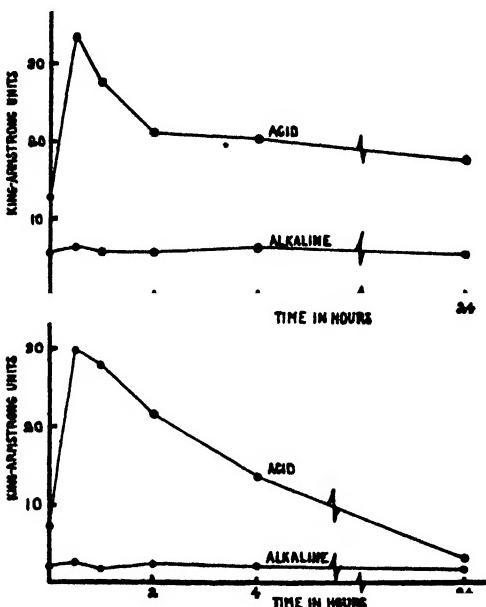


FIG. 1. Change in the serum phosphatase levels of rabbits following the intravenous injection of 5 ml. of 10% sodium taurocholate.

### Discussion

This work was undertaken in the hope that it might lead to an understanding of the mechanism of the increased alkaline phosphatase that undoubtedly occurs in the serum of both man (reviewed in 14, 18, 19) and experimental animals (5, 11, 24) after obstruction to the biliary tract. In this condition there is also a retention of surface-active bile salt in both man (23, 25) and experimental animals (6, 12, 17, 23). Since it has been shown that alkaline phosphatase is liberated from the white cell in the presence of bile salt *in vitro* (22) and also that the white cells of peripheral blood and other organs rich in white cells contain large amounts of alkaline phosphatase (9), it was possible that the retained bile salt might cause the increased alkaline phosphatase of obstructive jaundice by liberating the enzyme from the cells *in vivo*. However it was found that injection of bile salt into the blood stream of a rabbit in amounts far too small to produce a significant change in the plasma alkaline phosphatase activity produced an increase in the acid phosphatase activity. Since there is no increase in acid phosphatase in the sera of animals with biliary

obstruction (8), it is concluded that there is not sufficient retention of bile salt to explain the increase in alkaline phosphatase activity. The amount of bile salt that causes a rise in plasma acid phosphatase activity is much less than that necessary to produce a rise in alkaline phosphatase activity.

The acid phosphatase that appears in the plasma is possibly derived from the red cells. Red cells are known to be rich in phosphatase active at pH 4.5 to 5.5 (4, 16) and bile salt is known to lyse red cells. After an injection of bile salt the serum always showed evidence of some haemolysis. Lysis of 10% of the red cells would be sufficient to increase the plasma acid phosphatase some 20 King-Armstrong units per 100 ml., a rise of the same order as that found after the injection of bile salt.

Four theories have been advanced to explain the increase in serum alkaline phosphatase that occurs after biliary obstruction:

1. The liver produces more alkaline phosphatase than normal. Evidence for the hepatic origin of serum phosphatase has been put forward by Bodansky and Jaffe (5), Greene, Shattuck, and Kaplowitz (13), Freeman, Chen, and Ivy (11), and Oppenheimer and Flock (21).

2. The liver cannot excrete the normal amount of alkaline phosphatase and hence there is a retention of the enzyme in the blood. This view is held by Armstrong and King (2, 3) and evidence supporting the theory has been summarized by Gutman *et al.* (14). Both the histochemical studies of Wachstein and Zak (28) and the careful correlation of chemical and histochemical methods by Sherlock and Walshe (26) favor the retention theory.

3. The absence of bile in the intestine interferes with fat and calcium absorption. This produces an osteoporosis with a high serum alkaline phosphatase (Morris and Peden (20)). As Sherlock and Walshe (26) point out, this view would appear unlikely, since relief of the obstruction by a biliary fistula results in a fall in the serum phosphatase, although no bile has entered the intestine. Also return of the fistula bile to the gastrointestinal tract does not alter the rate at which the serum alkaline phosphatase decreases. Sherlock and Walshe (26) also claim that intravenous injection of calcium in subjects with obstructive jaundice does not reduce the serum alkaline phosphatase.

4. The sera of patients or animals with biliary obstruction contain an 'activator' which increases the activity of alkaline phosphatase already present (Thannhauser *et al.* (27)). It is claimed that the serum from a dog with biliary obstruction or the serum from a patient with a high serum phosphatase, when added to normal serum, increases its alkaline phosphatase activity. Evidence interpreted as favoring the 'activator' theory was also reported by Cantarow (7). On the other hand, Albers (1) could find no evidence of an 'activator', nor could Delory and King (10) in a series of experiments in which the hydrogen ion concentration was carefully controlled.

If the increased alkaline phosphatase that occurs in biliary obstruction were due to liberation of the enzyme from white cells, one would not expect an 'activation' in experiments, in which serum only was used, such as those

reported by Thannhauser *et al.* (27), and Delory and King (10), but one would expect an increase in the alkaline phosphatase of the plasma after the intravenous administration of bile salt. There is an increase in the acid phosphatase of the plasma which occurs after an injection far too small to produce an increase in the alkaline phosphatase. It is concluded, therefore, that the retention of bile salt does not explain the increase in serum alkaline phosphatase of biliary obstruction. These experiments lend no support to the 'activator' theory of Thannhauser *et al.* (27), even in a modified form.

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## ALKALINE AND ACID PHOSPHATASE IN CEREBROSPINAL FLUID

### DATA FOR NORMAL FLUIDS AND FLUIDS FROM PATIENTS WITH MENINGITIS, POLIOMYELITIS, OR SYPHILIS<sup>1</sup>

By K. G. COLLING AND R. J. ROSSITER

#### Abstract

Many normal cerebrospinal fluids contain an alkaline (pH 9.8) and an acid (pH 4.9) phosphatase. Both the alkaline and the acid phosphatase were significantly increased in the spinal fluids from patients with meningitis or poliomyelitis, but not in the fluids from patients with syphilis. The alkaline phosphatase activity was correlated with both the concentration of protein in the spinal fluid and with the white cell count, whereas the acid phosphatase was correlated with neither. When correction was made for the significant correlation between cell count and protein concentration, the partial correlation between alkaline phosphatase activity and both protein concentration and cell count remained significant statistically. In pathological conditions it appears likely that the alkaline phosphatase is derived partly from the polymorphonuclear leucocytes in the fluid and partly from the blood plasma. The acid phosphatase is probably derived from the lymphocytes of the fluid and possibly also from the blood plasma. It is unlikely that either of these enzymes comes from the substance of the brain or spinal cord. Acid phosphatase would be of more value than alkaline phosphatase as a diagnostic aid, since normal fluids contain much less of this enzyme.

#### Introduction

Previous publications from this laboratory have been concerned with the enzymes of white blood cells (6, 7, 18, 30, 31). Since pathological cerebrospinal fluids seemed a possible source of human white cells, a systematic study of the enzymes of spinal fluids from normal subjects and from patients with a number of diseases has been undertaken. Colling and Rossiter (5) have already reported that the true cholinesterase, but not the pseudocholinesterase, was significantly increased in the spinal fluids of patients with syphilis, while the pseudocholinesterase, and not the true cholinesterase, was increased in the fluids of patients with either meningitis or poliomyelitis. The true cholinesterase activity was correlated neither with the protein concentration nor with the cell count, while the pseudocholinesterase was correlated with the protein concentration and not with the cell count.

This paper concerns the phosphatases of normal and pathological cerebrospinal fluids. There are at least two phosphatases, or perhaps more correctly, phosphomonoesterases, in mammalian tissues (14, 29), an alkaline phosphatase with a pH optimum between 8.5-10, depending upon both the nature (26) and the concentration (6, 13, 20) of the substrate, and an acid phosphatase with a pH optimum between 4.5-5. Kaplan, Cohn, Levinson, and Stern (23), Fleischhacker (11), Kovács (28a), and Albers (1), all using a modification of the method of Bodansky (4), have reported the presence of a

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low concentration of alkaline phosphatase in normal cerebrospinal fluid. In certain pathological conditions the cerebrospinal alkaline phosphatase was increased. As far as we are aware there are no reports in the literature on the concentration of acid phosphatase in either normal or pathological cerebrospinal fluids.

### Methods

Phosphatase was measured by a modification of the method of King and Armstrong (25) in which the phenol, liberated from the hydrolysis of disodium phenyl phosphate, is determined by the method of Folin and Ciocalteu (12). Carbonate-bicarbonate buffer pH 9.8 (27) was used for the determination of the alkaline phosphatase and citrate buffer pH 4.9 (35) was used for the acid phosphatase. Because magnesium ions greatly activate many phosphatases (2, 6, 9, 13, 19, 21), the final concentration of magnesium in the reaction mixture was 0.001 *M*. This concentration of magnesium is optimal for many phosphatases (21).

To a 15 ml. centrifuge tube was added:—

- 2 ml. buffer (either pH 9.8 or pH 4.9)
- 2 ml. 0.01 *M* disodium phenyl phosphate
- 0.5 ml. 0.01 *M* magnesium chloride
- 0.2 ml. cerebrospinal fluid
- 0.3 ml. distilled water.

The tube was stoppered and incubated for 18 hr. in a 37° C. water bath. At the end of this time 1.8 ml. Folin and Ciocalteu reagent (diluted 1 : 3) was added and, after mixing, the tube was centrifuged. The phenol in 4 ml. of the supernatant was determined as described by King and Armstrong (25). A blank tube was also set up in which the substrate, buffer, and magnesium were incubated alone and the cerebrospinal fluid was added just before the Folin and Ciocalteu phenol reagent. The color of the test was read against that of the blank in a Coleman Universal Spectrophotometer at 650 m $\mu$ . The results were recorded as the quantity of phenol (in  $\mu$ gm.) liberated by 100 ml. cerebrospinal fluid in one hour. The figures for alkaline phosphatase can be approximately converted into the more familiar King-Armstrong units per 100 ml. (27) by dividing by 4000. Those for the acid phosphatase can be approximately converted into King-Armstrong units per 100 ml. (35) by dividing by 1000.

There is a theoretical objection to using such a long incubation period. The activity of the enzyme was not strictly linear with time for 18 hr. Since this lengthy incubation was necessary if measurable optical densities were to be obtained, some compromise seemed desirable. Dilution tests have shown that the enzyme activity, measured under the conditions of the experiment, was proportional to the concentration of the enzyme. Other workers on cerebrospinal fluid phosphatase have used incubation periods of as long as 24 hr. (23).

A drop of toluol was added to the total volume of cerebrospinal fluid immediately after it was collected. Both test and blank were always run in duplicate.

### Results

#### *Normal Cerebrospinal Fluids*

Table I shows the mean alkaline phosphatase activity of normal cerebrospinal fluids. These fluids were taken from apparently healthy adults under 55 years of age. The criteria of normality were the same as those described

TABLE I

ALKALINE PHOSPHATASE OF CEREBROSPINAL FLUID ( $\mu\text{GM. PHENOL 100 ML. HR.}$ )

Diagnosis	No. patients	Mean	S. E. mean	P
Normal	20	38	$\pm 13$	
Meningitis	13	608	$\pm 158$	<0.01
Poliomyelitis	24	98	$\pm 177$	<0.01
Syphilis, negative C. S. F. Wassermann	18	33	$\pm 10$	>0.7
Syphilis, positive C. S. F. Wassermann	11	73	$\pm 35$	>0.2

in our previous report (5). There was no history of systemic disease such as syphilis, tuberculosis, or diabetes. Each fluid had a normal protein concentration, cell count, and negative Wassermann and colloidal gold reactions. Many of the fluids were withdrawn from the subjects before the injection of a spinal anesthetic for a "cold" surgical operation. The presence of an alkaline phosphatase in some normal cerebrospinal fluids confirms the reports of other workers (1, 11, 23), but Table II shows that in 11 of the 20 normal fluids

TABLE II

DISTRIBUTION OF ALKALINE PHOSPHATASE IN CEREBROSPINAL FLUIDS

Diagnosis	Total No.	No. with alkaline phosphatase activity ( $\mu\text{gm. phenol}/100\text{ml./hr.}$ ) between:					
		0	1-50	51-100	101-150	151-200	200
Normal	20	11	5	1	1	2	
Meningitis	13			1	1	1	10
Poliomyelitis	24	3	3	9	5	2	2
Syphilis, negative C.S.F. Wassermann	18	9	3	4	2		
Syphilis, positive C.S.F. Wassermann	11	5	3	0	1		2

studied there was no hydrolysis of disodium phenyl phosphate at pH 9.8. In three out of these 20 fluids the alkaline phosphatase activity exceeded twice the standard deviation (Table III).

TABLE III

## ALKALINE PHOSPHATASE OF CEREBROSPINAL FLUID AND CLINICAL DIAGNOSIS

Diagnosis	No. patients	No. with normal alkaline phosphatase activity	No. with increased alkaline phosphatase activity	Percentage with increased alkaline phosphatase activity	$\chi^2$	P
Normal	20	17	3	15		
Meningitis	13	3	10	77	12.6	<0.01
Poliomyelitis	24	20	4	17	0.00	>0.9
Syphilis, negative C.S.F. Wassermann	18	18	0	0	0.00	>0.9
Syphilis, positive C.S.F. Wassermann	11	9	2	18	0.05	>0.7

Table IV shows the mean acid phosphatase activity of the normal fluids. By no means all of them hydrolyzed disodium phenyl phosphate at pH 4.9. Of the 20 normal fluids studied only six had a measurable acid phosphatase activity (Table VI).

*Meningitis*

Table I shows that the mean alkaline phosphatase activity of a group of fluids from patients with meningitis was much greater than that of the normal fluids. For the patients with meningitis there is a pronounced shift to the right in Table II, the alkaline phosphatase activity of 10 of the 13 fluids exceeding that of the most active normal fluid. The patients studied included two with meningococcal meningitis, three with pneumococcal meningitis, three with influenzal meningitis, and five with tuberculous meningitis. Table III shows that, of these 13 fluids, 10 had an increased alkaline phosphatase activity, i.e. an activity that exceeded the mean of the normal fluids by more than twice the standard deviation. A  $\chi^2$  test showed the increase in alkaline phosphatase activity of the meningitic fluids to be highly significant statistically ( $P < 0.01$ ).

The mean acid phosphatase activity of the fluids from patients with meningitis was also much greater than that of the normal fluids (Table IV). The fluids included two from patients with meningococcal meningitis, two from patients with pneumococcal meningitis, three from patients with influenzal meningitis, and two from patients with tuberculous meningitis. There appeared to be no definite relation between the enzyme activity and the type

TABLE IV

ACID PHOSPHATASE OF CEREBROSPINAL FLUID ( $\mu\text{GM. PHENOL}/100 \text{ ML./HR.}$ )

Diagnosis	No. patients	Mean	S. E. mean	P
Normal	20	5.5	$\pm 2.4$	
Meningitis	9	476	$\pm 323$	<0.05
Poliomyelitis	24	71	$\pm 25$	<0.02
Syphilis, negative C.S.F. Wassermann	16	12.9	$\pm 6.3$	>0.2
Syphilis, positive C.S.F. Wassermann	11	12.5	$\pm 7.4$	>0.2

of cell predominating in the spinal fluid, although it was noticed that the acid phosphatase activity was usually high in the fluids from patients with tuberculous meningitis where the predominating type of cell was the lymphocyte.

The scatter of the acid phosphatase values for the meningitic fluids was so great that the difference between the mean acid phosphatase activity of the meningitic and normal fluids was just significant statistically ( $P < 0.05$ ). This is brought out in Table V where it is seen that, of the nine fluids from patients with meningitis, two had no acid phosphatase activity whatsoever.

TABLE V  
DISTRIBUTION OF ACID PHOSPHATASE IN CEREBROSPINAL FLUIDS

Diagnosis	Total No.	No. with acid phosphatase activity ( $\mu\text{gm. phenol}/100 \text{ ml./hr.}$ ) between:				
		0	1 - 25	26 - 50	51 - 75	>76
Normal	20	14	4	2		
Meningitis	9	2		2		5
Poliomyelitis	24	7	2	5	3	7
Syphilis, negative C.S.F. Wassermann	16	12		2	2	
Syphilis, positive C.S.F. Wassermann	11	8	1		2	

One of these was from a patient with pneumococcal meningitis and one from a patient with influenzal meningitis. Both had an increased alkaline phosphatase activity.

Table VI shows that, of the nine fluids on which the acid phosphatase activity was determined, seven exceeded the mean of the normal fluids by more than twice the standard deviation. A  $\chi^2$  test showed this increase in acid phosphatase activity to be significant statistically ( $P < 0.01$ ).

TABLE VI  
ACID PHOSPHATASE OF CEREBROSPINAL FLUID AND CLINICAL DIAGNOSIS

Diagnosis	No. patients	No. with normal acid phosphatase activity	No. with increased acid phosphatase activity	Percentage with increased acid phosphatase activity	$\chi^2$	P
Normal	20	18	2	10		
Meningitis	9	2	7	78	13.3	<0.01
Poliomyelitis	24	9	15	63	12.7	<0.01
Syphilis, negative C.S.F. Wassermann	16	12	4	25	1.4	>0.2
Syphilis, positive C.S.F. Wassermann	11	9	2	18	0.4	>0.5

In many of the meningitic patients repeated spinal fluid examinations were made for alkaline and acid phosphatase activity. These revealed a similar increased activity but, with clinical improvement and decrease in spinal fluid cell count and protein concentration, the phosphatase activity decreased. The findings for the first test only for each patient is reported in the tables.

#### *Poliomyelitis*

The following observations were made on the spinal fluids from a group of patients with established poliomyelitis contracted during a mild poliomyelitis epidemic in the autumn of 1948. Table I shows that the mean alkaline phosphatase activity of the spinal fluids from patients with symptomatic poliomyelitis was significantly greater than that of the mean of the normal group ( $P < 0.01$ ). In Table II there is a distinct shift to the right for the fluids from patients with poliomyelitis, although the shift is not as pronounced as that observed for the fluids of those with meningitis. Because the scatter of the alkaline phosphatase activity of the control group was so great, only four of the 24 fluids from patients with poliomyelitis had an increased activity i.e. an activity which exceeded the mean of the control group by more than twice the standard deviation. A  $\chi^2$  test showed this not to be significantly different from the normal group.

The mean acid phosphatase activity of the spinal fluids from patients with poliomyelitis also was significantly ( $P < 0.02$ ) greater than the mean of the control group (Table IV). In Table V there is a shift to the right for the fluids from patients with poliomyelitis. Of the 24 patients with poliomyelitis, 15 had an increased acid phosphatase activity (Table VI). A  $\chi^2$  test showed this to be highly significant statistically ( $P < 0.01$ ). Of the nine fluids that failed to show an acid phosphatase activity greater than the mean of the normal group by more than twice the standard deviation, four were from patients who had passed the acute phase of their illness and who were detained

in hospital for physiotherapy. Thus, of the 20 patients with acute poliomyelitis, only five failed to show a significant increase in the spinal fluid acid phosphatase.

A group of seven undiagnosed patients were admitted to hospital during the poliomyelitis epidemic. These patients were suspected of having poliomyelitis. The clinical diagnosis ranged from 'influenza' to 'upper respiratory infection' and 'early subclinical poliomyelitis'. All had normal cerebrospinal fluid cell count and protein concentration. In the spinal fluids from five of these seven patients neither the alkaline nor the acid phosphatase activity was increased. Four of these five patients with normal spinal fluid phosphatase were subsequently discharged without developing poliomyelitis while the fifth developed acute rheumatic fever. There was a significant increase in both the alkaline and the acid phosphatase activity in the spinal fluid of the remaining two undiagnosed patients. On the basis of cerebrospinal fluid findings one of these was later diagnosed as having 'subclinical poliomyelitis' and the other subsequently developed a typical attack of acute symptomatic poliomyelitis.

### *Syphilis*

Table I gives the alkaline phosphatase activity of a series of fluids from a number of patients with syphilis (positive blood Wassermann reaction). The fluids were divided into two groups according as to whether the spinal fluid Wassermann reaction was positive or negative. Both Table I and Table II show that there was no significant increase in the alkaline phosphatase activity of either group, while Tables IV and V show that the same was true for the activity of the acid phosphatase.

### *Protein Concentration*

Table VII gives the coefficient of correlation between the concentration of protein in the spinal fluid and both the alkaline and the acid phosphatase

TABLE VII

CORRELATION BETWEEN ALKALINE AND ACID PHOSPHATASE ACTIVITY OF CEREBROSPINAL FLUIDS AND CONCENTRATION OF PROTEIN

Enzyme	Number of observations	Coefficient of correlation ( $r$ )	S.E. of $r$
Alkaline phosphatase	71	0.60	$\pm 0.08$
Acid phosphatase	69	0.11	$\pm 0.12$

activity. In compiling these statistics the fluids from all groups were pooled, regardless of clinical diagnosis. There was a statistically significant ( $r = 0.60 \pm 0.08$ ) correlation between the alkaline phosphatase activity and protein concentration, but that between acid phosphatase activity and protein concentration was not significant ( $r = 0.11 \pm 0.12$ ).

**Cell Count**

The alkaline phosphatase activity was also significantly ( $r = 0.75 \pm 0.05$ ) correlated with the number of cells in the cerebrospinal fluid (Table VIII), whereas the acid phosphatase activity was not ( $r = 0.03 \pm 0.12$ ).

TABLE VIII

CORRELATION BETWEEN ALKALINE AND ACID PHOSPHATASE ACTIVITY OF CEREBROSPINAL FLUIDS AND CELL COUNT

Enzyme	Number of observations	Coefficient of correlation ( $r$ )	S.E. of $r$
Alkaline phosphatase	71	0.75	$\pm 0.05$
Acid phosphatase	69	0.03	$\pm 0.12$

Thus the alkaline phosphatase activity was correlated with both the protein concentration and the cell count, while the acid phosphatase activity was correlated with neither. Table IX shows that, although there also was a significant correlation between cell count and protein concentration ( $r_{23} = 0.45 \pm 0.10$ ), the coefficient of partial correlation between alkaline phosphatase activity and protein concentration, with cell count excluded ( $r_{12.3} = 0.44 \pm 0.10$ ), was statistically significant. The same was true for the coefficient of

TABLE IX

CORRELATION COEFFICIENTS AND PARTIAL CORRELATION COEFFICIENTS BETWEEN ALKALINE PHOSPHATASE ACTIVITY, PROTEIN CONCENTRATION, AND CELL COUNT OF 71 CEREBRO-SPINAL FLUIDS

	Correlation between:	Coefficient of correlation ( $r$ )	S.E. of $r$
$r_{12}$	Alkaline phosphatase activity and protein concentration	0.60	$\pm 0.08$
$r_{13}$	Alkaline phosphatase activity and cell count	0.75	$\pm 0.05$
$r_{23}$	Protein concentration and cell count	0.45	$\pm 0.10$
$r_{12.3} = \frac{r_{12} - r_{12} \times r_{23}}{\sqrt{1 - r_{12}^2} \times \sqrt{1 - r_{23}^2}}$	Alkaline phosphatase activity and protein concentration (cell count excluded)	0.44	$\pm 0.10$
$r_{13.2} = \frac{r_{13} - r_{12} \times r_{23}}{\sqrt{1 - r_{13}^2} \times \sqrt{1 - r_{23}^2}}$	Alkaline phosphatase activity and cell count (protein concentration excluded)	0.68	$\pm 0.06$

partial correlation between the alkaline phosphatase activity and cell count with protein concentration excluded ( $r_{18.2} = 0.68 \pm 0.06$ ).

### Discussion

Many of the normal cerebrospinal fluids had both an alkaline and an acid phosphatase activity, but this finding was far from constant. A small percentage of the fluids had no phosphatase activity at all. The finding of an alkaline phosphatase in normal spinal fluid is a confirmation of the work of Kaplan *et al.* (23), Fleischhacker (11), Kovács (28a), and Albers (1).

It is well known that red blood cells contain an active acid phosphatase, sometimes of the order of 400 King-Armstrong units per 100 ml. packed cells (3, 28). A spinal fluid with 100 red cells per cu. mm. would thus have an acid phosphatase activity of approximately 4  $\mu\text{gm}$ . phenol per 100 ml. It is possible that contamination with blood at the time of lumbar puncture could account for a slight acid phosphatase activity in some of the fluids, although the low ( $5.5 \pm 2.4 \mu\text{gm}$ . phenol per 100 ml.) acid phosphatase activity of normal fluids would suggest that such contamination, if it occurs, is not great. All fluids obviously contaminated with blood were discarded. It is therefore extremely unlikely that the increase in acid phosphatase activity found in the spinal fluids of patients with meningitis or poliomyelitis can be explained in terms of red cell contamination.

The literature on the phosphatase activity of spinal fluids from patients with disease is not extensive. Kaplan *et al.* (23) reported an increase in the alkaline phosphatase activity of fluids from patients with purulent meningitis and a lesser increase in the fluids from patients with tuberculous meningitis, a result confirmed by Albers (1). Kaplan *et al.* (23) also reported a slight increase in the fluids from patients with poliomyelitis. Fleischhacker (11) found an increase in alkaline phosphatase activity in the fluids of 20 out of the 25 patients studied with neurosyphilis. Since he reported no figures and gave no definition of what he considered 'an increase' in alkaline phosphatase activity, it is possible that his figures were not significantly different from ours. Kovács (28a) also found that the alkaline phosphatase of the spinal fluid from patients with a number of diseases was increased.

As has been pointed out previously (5), Kaplan *et al.* (24) suggested that enzymes in normal cerebrospinal fluid may be derived from either the tissue of the central nervous system, i.e. brain or spinal cord, or from the blood plasma. They also suggested that the enzyme activity of the fluid from patients with disease could be increased by one or more of the following methods:—

- (a) An increase in enzyme-containing cells in the cerebrospinal fluid.
- (b) An increase in the permeability of the membrane between the blood plasma and the cerebrospinal fluid, with the passage of plasma enzymes into the cerebrospinal fluid.
- (c) A destruction of the tissue of the brain or spinal cord with the appearance of central nervous system enzymes in the cerebrospinal fluid.

### *Alkaline Phosphatase*

The finding that the alkaline phosphatase activity was correlated significantly with cell count, even when the protein concentration was excluded, suggests that this enzyme may be present in the white cells. This is to be expected when it is remembered that the white cells of both the rabbit (6, 7) and man (18) have a high alkaline phosphatase activity. This is sufficient to explain the increased alkaline phosphatase activity of the spinal fluids from patients with purulent meningitis observed by Kaplan *et al.* (23) and confirmed in our study. These workers also reported that the alkaline phosphatase activity of fluids from patients with purulent meningitis was greater than that of fluids from patients with tuberculous meningitis. This can be explained by the observation of Haight and Rossiter (18) that the polymorphonuclear leucocyte is rich in alkaline phosphatase, whereas the lymphocyte contains little of this enzyme.

That there was a slight, but very variable, increase in the alkaline phosphatase of spinal fluids from patients with poliomyelitis is probably explained by the variability of the type of cell commonly found in the cerebrospinal fluids of patients with this disease. Early in the disease, when polymorphonuclear leucocytes predominate, one would expect an increase in alkaline phosphatase activity, but later, when lymphocytes exceed polymorphonuclear leucocytes, one would expect this increase in alkaline phosphatase activity to disappear.

Unfortunately differential cell counts were not done because, at the time, the great importance of the type of cell was not realized. It seems clear that most of the increase in alkaline phosphatase activity of pathological fluids is brought about by Method (a), the type of cell responsible being the polymorphonuclear leucocyte, which is rich in this enzyme. A similar conclusion was reached by Kaplan *et al.* (24).

Normal cerebrospinal fluids may possess an alkaline phosphatase activity and yet contain no cells. Since the blood plasma is rich in alkaline phosphatase (14, 29), it is possible that some of this enzyme passes through the membrane between the blood plasma and the cerebrospinal fluid. Besides being correlated with the white cell count, the alkaline phosphatase activity was also significantly correlated with the concentration of protein in the spinal fluid, even when the cell count is excluded. In this respect it is similar to pseudocholinesterase. Each of these two enzymes is increased in the spinal fluids from patients with either meningitis or poliomyelitis and the concentration of each is significantly correlated with the concentration of protein in the fluid. Since pseudocholinesterase is present in the blood plasma and absent from both the white cells and the substance of the central nervous system ((5) for references), it is likely that this enzyme reaches the spinal fluid from the plasma. Such a suggestion has been made by Tower and McEachern (33, 34). Alkaline phosphatase may also reach the spinal fluid from the plasma in the same fashion. If this were so, the increased alkaline phosphatase of pathological spinal fluids, besides occurring by Method (a),

could also occur by Method (b). This was also suggested by Kaplan *et al.* (24) to explain the observation that, after removing the cells containing alkaline phosphatase by centrifuging, some alkaline phosphatase activity still remained. Method (b) might also explain the existence of alkaline phosphatase activity in normal spinal fluids containing no cells. Tower and McEachern, (33) reported that in spinal fluids from patients with craniocerebral trauma the pseudocholinesterase activity was increased. It would be interesting to know if there was also an increase in alkaline phosphatase in the fluids of patients with this condition.

Tissue of the central nervous system has been shown to contain an alkaline phosphatase by chemical methods (8, 10, 15) and histochemically (22) by the technique of Gomori (16) and Takamatsu (32). Tissue of the nervous system also contains a true cholinesterase, which was increased in the spinal fluids of patients with syphilis, a disease that produces evident damage in the central nervous system, but not in fluids from patients with meningitis or poliomyelitis (5). It was suggested that the increased amounts of this enzyme in the spinal fluids of patients with syphilis came from the substance of the nervous system. Unlike true cholinesterase, there was no significant increase in the alkaline phosphatase activity of fluids from patients with syphilis, although there was an increase in fluids from patients with meningitis or poliomyelitis. It would thus seem unlikely that the increase in alkaline phosphatase activity in the fluids of patients with these diseases is brought about by Method (c).

#### *Acid Phosphatase*

For both man and the rabbit the concentration of acid phosphatase is high in the lymphocyte and low in the polymorphonuclear leucocyte. Also for man the activity of the acid phosphatase in the lymphocyte is approximately eight times that of the alkaline phosphatase in the polymorphonuclear leucocyte (18). One would expect, therefore, an increase in acid phosphatase activity in conditions where there is an increase in lymphocytes in the cerebro-spinal fluid. This increase should be all the more pronounced since the activity of the acid phosphatase of normal fluids is very much less than that of the alkaline phosphatase. In conditions where there is an increase in the number of lymphocytes in the spinal fluid (e.g. meningitis and poliomyelitis) there was a very significant increase in acid phosphatase activity. The activity of the acid phosphatase, unlike that of the alkaline phosphatase, was not correlated significantly with the total cell count, but this is to be expected since in most instances the majority of the cells were polymorphonuclear leucocytes. The acid phosphatase was present in a few cells at a high concentration, whereas the alkaline phosphatase was present in many cells at a lower concentration. It seems likely that most of the acid phosphatase in pathological fluids is accounted for by Method (a).

Acid phosphatase is found in quite high concentration in blood plasma (14, 29). Also the acid phosphatase was increased in fluids from patients

with meningitis or poliomyelitis, conditions in which there was also an increase in the spinal fluid alkaline phosphatase and pseudocholinesterase, two enzymes part or all of which, it has been suggested, comes from the blood plasma. Since the acid phosphatase activity, unlike that of the alkaline phosphatase or pseudocholinesterase, is not correlated with the protein concentration, Method (b) would appear to be of less importance for acid phosphatase. The method cannot be excluded, however, and may be the chief source of acid phosphatase in those normal fluids that have enzyme activity.

Acid phosphatase has also been demonstrated in the substance of the central nervous system by chemical methods (8, 10, 15) and histochemically (36) by the method of Gomori (17) but, for the same reasons advanced for alkaline phosphatase, it is considered unlikely that the acid phosphatase is increased by Method (c).

From the point of view of diagnosis, cerebrospinal fluid acid phosphatase would appear to be of value in poliomyelitis. Because of the wide scatter in the control group, satisfactory conclusions cannot be drawn from alkaline phosphatase estimations. Both alkaline and acid phosphatase determinations could be used as an aid in the diagnosis of meningitis. However these tests have no obvious advantages over the established diagnostic procedures for this disease. The test would appear to be of no value in the diagnosis of syphilis of the nervous system.

This work must be considered as preliminary, and of an exploratory nature only. Before phosphatase estimations could be recommended as a routine diagnostic procedure a full investigation of spinal fluids from patients with diseases of the central nervous system other than those already investigated would have to be undertaken. In addition a careful study of the changes of the phosphatase activity of the spinal fluid from the same patient throughout the entire course of such diseases would be highly desirable.

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## **$\beta$ -GLUCURONIDASE OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES<sup>1</sup>**

By R. J. ROSSITER AND ESTHER WONG

### **Abstract**

Rabbit polymorphonuclear leucocytes contain an enzyme capable of hydrolyzing biosynthetic phenolphthalein mono- $\beta$ -glucuronide. The concentration of the enzyme in the white cell is some 2000 times the concentration of the enzyme in the blood plasma. Under the conditions of study, the  $\beta$ -glucuronidase activity was proportional to the concentration of the enzyme. The effect of substrate concentration on the enzyme activity was studied and the Michaelis constant,  $K_m$ , determined. The course of the reaction was linear with time for the first 12 hr. and then fell off slightly during the next 12 hr. The optimum pH of the enzyme was 4.45 in either 0.2 M acetate or 0.2 M phthalate buffer. It was not inhibited by cyanide, azide, iodoacetate, fluoride, glycine, thiourea, urethane, arsanilic acid, acetophenone, *o*-cresol or *m*-cresol, in a final concentration of 0.01 M. The possible function of  $\beta$ -glucuronidase in rabbit polymorphonuclear leucocytes is discussed.

### **Introduction**

Rabbit polymorphonuclear leucocytes contain a phosphomonoesterase (Cram and Rossiter (1, 2)) and an ali- or common esterase (Rossiter and Wong (35, 36)). It has now been shown that these cells also contain a  $\beta$ -glucuronidase capable of hydrolyzing phenolphthalein mono- $\beta$ -glucuronide.

In 1914 Sera (38) showed that the liver, kidney, and spleen of the ox, rabbit, and dog contain an enzyme that is able to hydrolyze glucuronides. Subsequently the enzyme was partially purified by Masamune (26), Oshima (32), and others (4, 10, 12, 17). In 1946 Fishman introduced a convenient method for determining the activity of  $\beta$ -glucuronidase, based on the liberation of free phenolphthalein, colored at pH greater than 10, from biosynthetic phenolphthalein mono- $\beta$ -glucuronide (39). This determination and the method of preparation of the substrate was later improved (9).

Fishman, Springer, and Brunetti (9) reported that there was considerable  $\beta$ -glucuronidase activity in human blood and that much of this was in the buffy-coat layer. Rossiter and Wong (37) showed that for man the enzyme was present in both the polymorphonuclear leucocyte and the lymphocyte, the ratio being of the order of 5 : 4. In the present paper, some of the properties of the  $\beta$ -glucuronidase of rabbit polymorphonuclear leucocytes are described.

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## Methods

### Cell Preparation

Polymorphonuclear leucocytes were obtained from the rabbit by the method of de Haan (13), the details of which have already been described by Cram and Rossiter (2). At least 95% of the cells were polymorphonuclear leucocytes, the remainder being lymphocytes or monocytes.

### $\beta$ -Glucuronidase Determination

The glucuronidase activity was determined by the method of Fishman *et al.* (9) using the substrate prepared as described by these workers. To a centrifuge tube was added:—

- 0.2 ml. cell suspension
- 0.8 ml. 0.1 M acetate buffer, pH 4.5
- 0.1 ml. 0.01 M phenolphthalein mono- $\beta$ -glucuronide
- 0.2 ml. 0.5% saponin.

The tube was placed in a 38° C. water bath for 2–16 hr. at the end of which time the reaction was stopped by the addition of 1 ml. 10% trichloroacetic acid. A control tube containing enzyme, buffer, and saponin was incubated for the same length of time and the substrate was added just before the trichloroacetic acid. The determination was completed as described by Fishman *et al.* (9), the test and control being read against a blank in a Coleman universal spectrophotometer at 540 m $\mu$ . A standard containing 20  $\mu$ gm. phenolphthalein per tube was read with each set of determinations. The test was always run in duplicate.

The object of the saponin was to extract the enzyme from the cell. Rossiter (33) showed that certain surface-active substances, such as saponin, alkyl sulphate, or bile salts, liberate the enzymes phosphomonoesterase and alisterase from rabbit polymorphonuclear leucocytes into the surrounding fluid. It is shown in this paper that saponin also liberates  $\beta$ -glucuronidase from these cells.

### Recording of Results

Enzyme activity has been recorded in terms of glucuronidase units per 100 ml. cell suspension where, following Fishman (9, 39), one glucuronidase unit is defined as the amount of enzyme that would liberate one  $\mu$ gm. phenolphthalein in one hour under the standard conditions of the test. To bring the values for white-cell glucuronidase into line with those of other white-cell enzymes studied in the laboratory, the results have also been given in terms of glucuronidase units per  $10^{10}$  cells. This has the further advantage that  $10^{10}$  packed cells has a wet weight in the neighborhood of 4 gm. (34). It is thus possible to compare the concentration of the enzyme in the leucocyte with that in other tissues.

## Results

### Concentration of $\beta$ -Glucuronidase in Rabbit Polymorphonuclear Leucocytes

Table I gives the concentration of  $\beta$ -glucuronidase in rabbit polymorphonuclear leucocytes and Table II gives the concentration of the enzyme in

TABLE I  
 $\beta$ -GLUCURONIDASE ACTIVITY OF SUSPENSIONS OF RABBIT POLYMORPHONUCLEAR  
 LEUCOCYTES

Rabbit No.	Units/ $10^{10}$ cells	Units/100 gm. tissue
1	8600	215,000
2	13,300	332,000
3	21,100	527,000
4	10,800	270,000
5	9900	248,000
6	11,100	277,000
7	9100	238,000
8	9100	238,000
9	8900	223,000
10	11,800	295,000
14	4600	115,000
15	7700	192,000
16	5600	140,000
17	6500	165,000
18	7900	198,000
Mean ( $\pm$ S.D.) =	9700 ( $\pm$ 3800)	243,000 ( $\pm$ 94,000)

TABLE II  
 $\beta$ -GLUCURONIDASE ACTIVITY OF RABBIT PLASMA

Rabbit No.	Units/100 ml.	Rabbit No.	Units/100 ml.
1	127	5	67
2	131	12 R2	67
3	113	13 RB1	226
4	117	8 RX	76
5 R1	203	9 RX	9

Mean ( $\pm$  S.D.) = 114  $\pm$  62

rabbit plasma. The mean ( $\pm$  S.D.) enzyme activity of the white cells was 9700 ( $\pm$  3800) units per  $10^{10}$  cell or 243,000 units per 100 gm. tissue. The mean activity of the white cells was thus some 2000 times greater than that of the rabbit plasma, 114 ( $\pm$  62) units per 100 ml.

#### *The Extraction of Enzyme from Cell*

Fishman *et al.* (9) extracted  $\beta$ -glucuronidase from white blood cells by the alternate freezing and thawing of the cells. Since it had previously been shown that other enzymes could be extracted by surface-active substances (33), the effect of adding saponin to suspensions of rabbit polymorphonuclear leucocytes was investigated (Table III). One sample of cells was suspended in isotonic saline and a similar sample in 0.5% saponin in isotonic saline. After the  $\beta$ -glucuronidase activity had been determined in each, the two suspensions were centrifuged. The resulting cell-containing residues were made up to

their original volume with isotonic saline. The  $\beta$ -glucuronidase activity of each of the two residues and each of the two cell-free supernatants was then determined.

The first column of Table III shows that when saponin was added to the cells the activity of the suspension was greater. A similar finding was reported for phosphomonoesterase (2, 33). Presumably, because of the limited permeability of the cell membrane to phenolphthalein mono- $\beta$ -glucuronide, the substrate did not saturate all the active centers of the enzyme within the cell. When saponin was added, substrate was available to all of the enzyme and maximum activity resulted. That the enzyme was liberated from the cell is shown by the second and third columns of Table III. For the cells originally

TABLE III

EFFECT OF 0.5% SAPONIN ON THE DISTRIBUTION OF  $\beta$ -GLUCURONIDASE BETWEEN CELLS AND SUPERNATANT OF SUSPENSIONS OF WASHED RABBIT POLYMORPHONUCLEAR LEUCOCYTES

Cells suspended in	Glucuronidase activity (units/100 ml.)		
	Original suspension	Supernatant	Cells
Isotonic saline	244	34	185
0.5% saponin in isotonic saline	266	249	13
Isotonic saline. Cells centrifuged off and resuspended in 0.5% saponin in isotonic saline	—	—	244

suspended in isotonic saline, the greater part of the enzyme activity, 185 units per 100 ml., remained in the cell-containing residue and only a little, 34 units per 100 ml., was in the supernatant. For the cells originally suspended in 0.5% saponin in isotonic saline, the greater part of the activity, 249 units per 100 ml., was in the cell-free supernatant, only 13 units per 100 ml. remaining in the cells. Moreover, when 0.5% saponin in isotonic saline was added to cells that previously had been suspended in saline only and separated by centrifuging, the activity was increased from 185 units per 100 ml. to 244 units per 100 ml., an increase similar to that observed when saponin was added to the original suspension.

The effect of adding 0.2 ml. 0.5% saponin to the reaction mixture containing 0.2 ml. cell suspension was compared with the freezing-thawing method in the experiments reported in Table IV. Although freezing and thawing did increase the enzyme activity of a suspension of cells in water, the effect of adding 0.2 ml. 0.5% saponin to the reaction mixture was much greater. The addition of saponin to the cells that previously had been frozen and thawed produced little more activity than did the addition of saponin to cells that had not been subjected to the freezing-thawing procedure.

TABLE IV

COMPARATIVE EFFECTS OF SAPONIN AND ALTERNATE FREEZING AND THAWING ON THE  $\beta$ -GLUCURONIDASE ACTIVITY OF SUSPENSIONS OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES

Additions to reaction mixture	Glucuronidase activity (units/100 ml.)	
	Preparation I	Preparation II
0.2 ml. water	614	279
0.2 ml. 0.5% saponin	935	353
0.2 ml. water cells frozen and thawed nine times	746	329
0.2 ml. 0.5% saponin cells frozen and thawed nine times	990	347

Table V shows that, for the cell suspensions used, 0.2 ml. 0.5% saponin produced a maximal effect. Concentrations of saponin greater than 1% were slightly inhibitory, while concentrations less than 0.5% were not sufficient to liberate all the enzyme from the cells.

TABLE V

EFFECT OF SAPONIN ON THE  $\beta$ -GLUCURONIDASE ACTIVITY OF SUSPENSIONS OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES

Additions to reaction mixture	Glucuronidase activity (units/100 ml.)	
	Preparation I	Preparation II
0.2 ml. water	350	536
0.2 ml. 10% saponin	338	518
0.2 ml. 5% saponin		539
0.2 ml. 1% saponin	412	575
0.2 ml. 0.5% saponin	423	592
0.2 ml. 0.1% saponin	417	560
0.2 ml. 0.01% saponin	—	508

Sodium taurocholate ( $10^{-3} M$ ) had an effect similar to that of saponin.

#### Enzyme Concentration

Under the conditions of study, the  $\beta$ -glucuronidase activity of a preparation of rabbit polymorphonuclear leucocytes was proportional to the concentration of the enzyme (Fig. 1).

#### Substrate Concentration

Fig. 2 shows the effect of the concentration of the substrate on the hydrolysis of phenolphthalein glucuronide by the rabbit polymorphonuclear-leucocyte enzyme. The curve is the typical hyperbola of an enzyme whose enzyme-substrate relation can be described in terms of Michaelis-Menten theory. Substrate concentrations of  $0.001 M$  or above produced maximum enzyme activity. In contrast to the findings of Talalay *et al.* (39) for a partially

purified enzyme preparation from mouse spleen, liver, and kidney, excess of the substrate was not inhibitory for substrate concentrations up to 0.0022 M. Higher substrate concentrations were not tested. With other substrates and

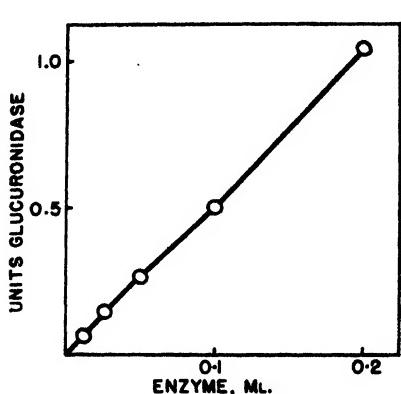


FIG. 1.

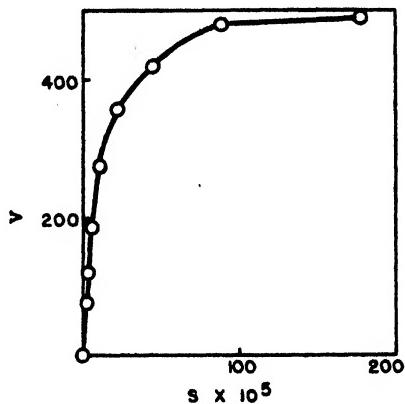


FIG. 2.

FIG. 1. *The relation between enzyme activity and enzyme concentration of rabbit polymorphonuclear leucocyte  $\beta$ -glucuronidase.*

FIG. 2. *The effect of substrate concentration on the initial velocity of rabbit polymorphonuclear leucocyte  $\beta$ -glucuronidase. V is the initial velocity in mgm. phenolphthalein per 100 ml. per hr. and S is the substrate concentration in gm. molecules per liter. Substrate, phenolphthalein mono- $\beta$ -glucuronide. Temperature, 38° C.*

other enzyme sources, inhibition of  $\beta$ -glucuronidase by excess substrate has been described (5, 18).

Fig. 3 is a plot of  $1/V$  against  $1/S$  according to Lineweaver and Burk (24). The Michaelis constant,  $K_m$ , of four different preparations of the enzyme from

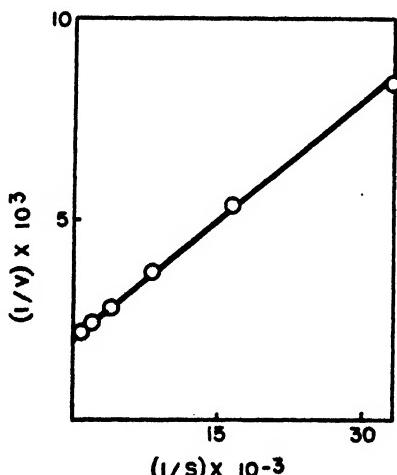


FIG. 3. *Plot of  $1/V$  against  $1/S$  for rabbit polymorphonuclear leucocyte  $\beta$ -glucuronidase. V is the initial velocity in mgm. phenolphthalein per 100 ml. per hr. and S is the substrate concentration in gm. molecules per liter.*

polymorphonuclear leucocytes was 0.000093 M, 0.000100 M, 0.000111 M, and 0.000117 M, respectively. These figures are slightly higher than the values of 0.000053 M and 0.000045 M for the  $K_s$  of the  $\beta$ -glucuronidase from mixed mouse organs reported by Talalay *et al.* (39) using the same substrate, buffer, and hydrogen ion concentration. The values of  $K_s$  for phenolphthalein glucuronide are much lower than those reported in the literature for other substrates, 0.0005 M for oestriol glucuronide, 0.004 M for menthyl glucuronide, 0.01 M for borneol glucuronide with ox-spleen  $\beta$ -glucuronidase (5), and 0.0035 M for phenyl glucuronide with mouse-liver enzyme (18).

#### *Course of Reaction with Time*

Under the conditions of study, the rate of hydrolysis of phenolphthalein glucuronide was reasonably constant with time for the first 12 hr. During the second 12 hr., the decrease in activity was slight (Fig. 4). Table VI shows that if the glucuronidase activity of an enzyme preparation is determined using incubation periods up to 12 hr. the results are satisfactory. The result for a 24-hr. incubation period is some 10% too low.

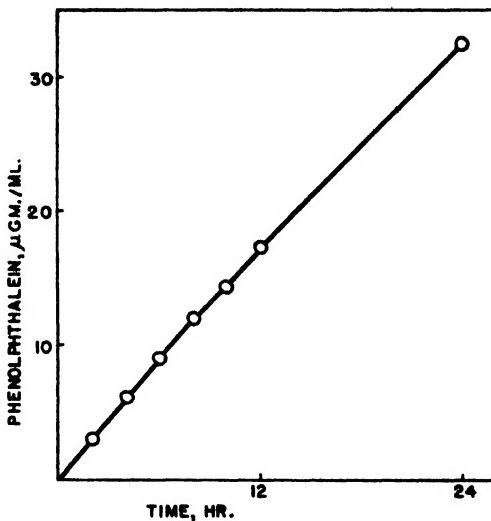


FIG. 4. Time course of the reaction for rabbit polymorphonuclear leucocyte  $\beta$ -glucuronidase. Substrate, phenolphthalein mono- $\beta$ -glucuronide. Temperature, 38° C.

#### *Hydrogen Ion Concentration*

The optimum hydrogen ion concentration was 4.45 in 0.2 M acetate buffer (Fig. 5). A similar figure was obtained when 0.2 M phthalate buffer was used. This agrees with the values found by Talalay *et al.* (39) using phenolphthalein glucuronide as substrate, although other workers employing different substrates and different enzyme sources have reported pH optima varying from 4.3 to 5.6 (5, 18, 26, 32). Mills (28) separated ox-spleen  $\beta$ -glucuronidase into two fractions, one having an optimum pH of 4.5 for menthyl, phenyl, and phenolphthalein glucuronide and one having an optimum pH of 5.0-5.2 for

the same three substrates. Fig. 5 shows that in the preparation used, there was no suggestion of a peak at pH 5.0-5.2. Kerr *et al.* (18) reported that the  $\beta$ -glucuronidase of mouse liver and spleen also had two pH optima, one in

TABLE VI

COURSE OF REACTION WITH TIME. ENZYME,  $\beta$ -GLUCURONIDASE OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES, SUBSTRATE, PHENOLPHTHALEIN MONO- $\beta$ -GLUCURONIDE  
TEMPERATURE, 38° C.

Time (hr.)	Phenolphthalein liberated ( $\mu\text{gm}./\text{ml. reaction mixture}$ )	Glucuronidase activity (units/ $10^{10}$ cells)
2	3.0	970
4	6.1	990
6	9.0	970
8	12.0	970
10	14.2	920
12	17.3	930
24	32.3	880

the region of pH 4.5 and one in the region of pH 5.2. Subsequently these workers showed (17) that the same was true for mouse-kidney  $\beta$ -glucuronidase, but not for the enzyme from the mouse uterus. It would thus appear that, on the basis of the pH-activity curve, the  $\beta$ -glucuronidase of rabbit polymorphonuclear leucocytes resembles that of the mouse uterus.

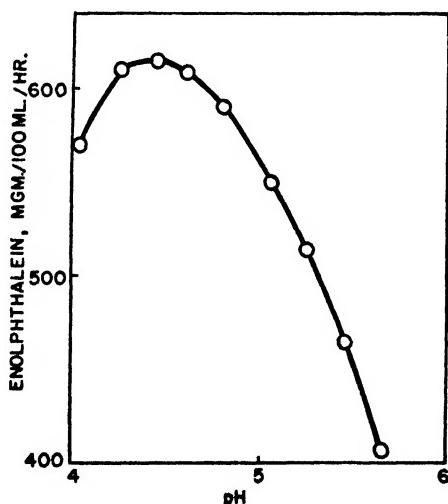


FIG. 5. The effect of hydrogen ion concentration on the activity of rabbit polymorphonuclear leucocyte  $\beta$ -glucuronidase. Substrate phenolphthalein mono- $\beta$ -glucuronide. Temperature, 38° C. Buffer, 0.2 M acetate.

### Inhibitors

Glucuronidase is not inhibited by many of the usual enzyme inhibitors. The following substances produced negligible inhibition when present in the reaction mixture in a final concentration of 0.01 M: cyanide, azide, iodoacetate, fluoride, glycine, thiourea, urethane, arsanilic acid, acetophenone, *o*-cresol, and *m*-cresol. Of these substances, fluoride (0.015 M), thiourea (0.0015 M), and urethane (0.015 M) have been shown to have no effect on mouse-liver  $\beta$ -glucuronidase (16).

### Discussion

The distribution of  $\beta$ -glucuronidase in the animal body has been studied by Oshima (31) and Talalay *et al.* (39). Both groups of workers found high enzyme activity in the spleen. Because of the limited amount of material available no attempt was made to purify the enzyme from the rabbit polymorphonuclear leucocytes. If, as has now been shown for ox-spleen and other organs (17, 18, 28, 29), it were found that the rabbit polymorphonuclear leucocyte contained two or more enzymes, it is possible that the kinetic data would have to be reinterpreted. As has been pointed out above, a study of the pH-activity curve for the crude rabbit polymorphonuclear leucocyte enzyme shows little evidence of more than one maximum.

The high concentration of  $\beta$ -glucuronidase found in the polymorphonuclear leucocyte of the rabbit and also in the white cells of man (9, 37) raises the question of whether the white cells might not be an important source of plasma  $\beta$ -glucuronidase. But, as was pointed out for phosphomonoesterase (2), the possibility still remains that the white cell may have obtained its  $\beta$ -glucuronidase by adsorption of the enzyme from the plasma. If this were so, the white cells must have the extraordinary ability of concentrating the enzyme by a factor of 2000, for the concentration of  $\beta$ -glucuronidase was of the order of 250,000 units per 100 gm. packed white cells compared with 115 units per 100 ml. rabbit plasma. It seems likely that the enzyme is within the cell rather than adsorbed on the cell surface, for it has been demonstrated histochemically by Friedenwald and Becker (11) in the cells of the spleen, lymphatic nodules, and bone marrow. The enzyme was in the cytoplasm rather than in the nucleus.

It is interesting to speculate upon the function of  $\beta$ -glucuronidase in polymorphonuclear leucocytes, especially since it has been suggested that  $\beta$ -glucuronidase has to do with metabolic conjugation and, possibly, detoxication (6, 7, 31). Polymorphonuclear leucocytes quickly aggregate at sites of infection. It would be attractive to assume that here they inactivated toxic substances by conjugation. The theory that  $\beta$ -glucuronidase is concerned with metabolic conjugation and detoxication has, however, been severely criticized (15, 16, 21, 22). The chief function of the enzyme appears to be hydrolytic (27, 30). It is clear from the work of Levvy and his associates that the *in vitro* synthesis of glucuronides is brought about by a different

enzyme system from the  $\beta$ -glucuronidase that hydrolyzes biosynthetic glucuronides (21, 23). Inhibition of glucuronide synthesis does not inhibit glucuronide hydrolysis (3, 14, 25) nor does the reduction of glucuronide hydrolysis by either the *in vitro* (16) or the *in vivo* (15) administration of inhibitors decrease glucuronide synthesis. Also, if glucuronide hydrolysis is increased, there is no increase in glucuronide synthesis (15).

The chief proponent of the conjugation theory is Fishman. He showed that the injection of menthol to dogs and mice caused an increase in the  $\beta$ -glucuronidase of the liver, spleen, and kidney, with no change in the uterus (6). Subsequently he showed that the administration of oestrogens to ovariectomized mice produced an increase in the  $\beta$ -glucuronidase of the uterus, but no change in that of the liver (7, 8). It was suggested that the increase in the enzyme following menthol administration was evidence for the view that the enzyme has to do with the conjugation of menthol and that the increase in the uterus following oestrogen administration was evidence that it has to do with metabolic conjugation. Levvy and co-workers, on the other hand, believe that the  $\beta$ -glucuronidase activity of an organ parallels the degree of tissue regeneration and cell proliferation (19, 20, 22). They also suggested that the rise in the uterine  $\beta$ -glucuronidase after the administration of oestrogens could also be explained by cell proliferation.

If the function of  $\beta$ -glucuronidase is concerned with the hydrolysis of glucuronides rather than their synthesis, the role of the enzyme in the white cell may be concerned with one of the stages in the hydrolysis of a substance normally present in tissues that contain glucuronic acid e.g. hyaluronic acid or chondroitin sulphuric acid (9). It is thus conceivable that the white-cell enzyme may play some part in the complex tissue reaction of inflammation.

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## USE OF OLEIC ACID - ALBUMIN AGAR MEDIUM FOR THE STUDY OF STREPTOMYCIN RESISTANCE OF *MYCOBACTERIUM TUBERCULOSIS*<sup>1</sup>

By CHARLES O. SIEBENMANN<sup>2</sup>

### Abstract

The oleic acid - albumin agar, proposed by Dubos and Middlebrook as a diagnostic medium for the isolation of *Mycobacterium tuberculosis*, proved useful for determining streptomycin resistance. By growing cultures of *M. tuberculosis* on this translucent substrate, containing graded amounts of streptomycin, a close correlation was found between the observed streptomycin resistance and that determined in fluid tween-albumin medium. The initial presence of a few organisms, showing a slight resistance to streptomycin, was found not to be an exclusive characteristic of cultures developing drug resistance in the course of streptomycin therapy. Cultures not developing resistance may also contain such resistant cells.

The use of Dubos fluid medium (2) for the determination of streptomycin resistance of *Mycobacterium tuberculosis* has been adopted by many clinical laboratories as a convenient and relatively rapid method, yielding reproducible results. In the case of streptomycin-sensitive cultures, this method fails, however, to reveal the presence of streptomycin-resistant tubercle bacilli if their relative numbers are small (9). That such streptomycin-resistant cells may be present even in cultures which were never in contact with streptomycin was demonstrated for the first time by Pyle (6) who seeded such cultures on Herrold's egg yolk - agar medium containing graded amounts of streptomycin. This medium has been used successfully for determining streptomycin resistance (1, 6, 8). For investigational purposes in the field of chemotherapy, this medium has, however, some disadvantages. The presence of egg yolk makes it chemically complex and over-rich in nutrient material, which tends to mask antibacterial activity of chemotherapeutic agents to be tested. As a medium more suited for our purposes, we chose oleic acid - albumin agar, proposed by Dubos and Middlebrook (2) for the isolation of *M. tuberculosis*. Its usefulness as a diagnostic medium was confirmed by Smith *et al.* (7). It is chemically well defined and permits rapid, though not over-abundant growth. As a translucent medium, it lends itself ideally to the microscopic study of the colonial characteristics of *M. tuberculosis* grown in the presence or absence of chemo-therapeutic agents.

In the following study the use of this medium is described for the determination of streptomycin sensitivity of human strains of *M. tuberculosis*. By means of this technique, cultures of *M. tuberculosis*, isolated prior to the start of streptomycin therapy, were tested for the possible presence of streptomycin-resistant cells. The purpose of this study was to investigate whether

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Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario.

<sup>2</sup> With the technical assistance of W. H. Linklater and R. Otlakowski.

the presence of such resistant cells could account for the later development of streptomycin resistance.

### Methods

Oleic acid - albumin agar was prepared essentially according to the authors' description (2). The streptomycin was incorporated in the form of streptomycin sulphate, added as an aqueous solution, simultaneously with the oleic acid - albumin complex. This addition was made after the basal medium was cooled to 50° C. Slants were poured, using 1 oz. flat, rectangular vials with bakelite screw caps. The usable agar surface was 3 X 6 cm.

The following concentrations of streptomycin were used: 0, 0.5, 1, 10, and 100 µgm. per ml. of medium.

The growth observed on oleic acid - albumin agar was compared with the growth obtained after seeding the cultures on Loewenstein - Jensen (5) slopes contained in similar vials.

For testing cultures of *M. tuberculosis* for streptomycin sensitivity, using oleic acid - albumin agar, these were first grown for 10 days on Dubos fluid medium. For seeding the agar slants, a fivefold dilution of such cultures in Dubos medium was used. Each slant was seeded with two inocula with a 5 mm. platinum loop. Control counts, made in some instances on oleic acid - albumin agar plates, indicated that such seedings contained in the order of 50—100,000 viable cells.

Of some 20 cultures of *M. tuberculosis* seeded on these oleic acid - albumin agar slants all grew well and more rapidly, though less abundantly, than on Loewenstein-Jensen slopes, first growth generally appearing within one week. Final readings were made after six weeks. Little change was noticed after the fourth week of incubation (38-39° C.).

When using fluid Dubos medium (containing 0.05% tween 80) for determining streptomycin sensitivity the technique recommended by the Department of Veterans' Affairs\* was followed in all essential features. Streptomycin sensitivity is defined as the lowest concentration of streptomycin which inhibits the growth of a culture during a 14 day period of incubation.

### Presence of Partially Resistant Cells in Otherwise Streptomycin-Sensitive Cultures

Table I records observations made on two resistant and one partially resistant strain of *M. tuberculosis*. For comparison the three original, streptomycin-sensitive strains, from which the streptomycin-resistant variants were derived, were examined. In the first culture (H37RV) the resistance was produced by prolonged *in vitro* exposure of the culture to streptomycin. In the two other cultures (67 and 913) resistance developed *in vivo* in the course of streptomycin therapy of the patient.

\* Personal communication, courtesy of Dr. Marion Ross, Christie Street Hospital, Toronto.

TABLE I  
STREPTOMYCIN SENSITIVITY OF HUMAN STRAINS OF *M. tuberculosis*

Cultures***	Fluid Dubos medium**	Colony counts on streptomycin oleic acid agar*				
		0	0.5	1	10	100
H37RV	0.2	Innum.	0	0	0	0
H37RV Res.	>1024	Innum.	Innum.	Innum.	Innum.	Innum.
15	0.2	Innum.	100	0	0	0
67 Res.	>1024	Innum.	Innum.	Innum.	Innum.	Innum.
710	0.2	Innum.	6	0	0	0
913 Res.	1.0	Innum.	Innum.	Innum.	0	0

\* Containing designated amounts of streptomycin expressed in  $\mu\text{gm. per ml.}$  of medium.

\*\* Figures record minimum bacteriostatic concentrations of streptomycin in  $\mu\text{gm. per ml.}$ .

\*\*\* Received from The Medical School, Northwestern University, Chicago (Courtesy of Dr. G. P. Youmans.).

The streptomycin sensitivities of these six cultures (Table I), as determined in fluid Dubos medium (Column 2), broadly agrees with the growth observations made on oleic acid - albumin agar (Columns 3-7). In the case of streptomycin-sensitive cultures, the advantage of the solid substrate over the fluid medium becomes evident in that it reveals the presence of colonies derived from cells of increased streptomycin resistance.

To investigate whether the presence, in some cultures of *M. tuberculosis*, of a small number of resistant cells could account for the later development by these cultures of a marked drug resistance, the following series of experiments were carried out.

Two groups of pretreatment cultures of *M. tuberculosis* were examined for the presence of cells of increased streptomycin resistance:

*Group A* comprised of cultures obtained from patients from whom, after streptomycin therapy, drug-resistant cultures were isolated.

*Group B* cultures isolated from patients from whom, after streptomycin treatment, only streptomycin-sensitive cultures were obtained.

For these two groups of cultures streptomycin sensitivity was determined on oleic acid - albumin agar as described above. The results (see Table II) show that cultures containing cells of partial streptomycin resistance are found in both groups of cultures, though they are more frequent among the group which developed drug resistance in the course of streptomycin therapy. In this latter group all five cultures showed colonies in the presence of 0.5  $\mu\text{gm.}$  of streptomycin, whereas in the group which remained resistant only four out of eight cultures contained such cells.

### Discussion and Conclusions

From the experimental data presented it seems evident that in the case of a culture of *M. tuberculosis*, isolated prior to the start of streptomycin treatment, the presence of a few cells showing increased streptomycin resistance

TABLE II

STREPTOMYCIN SENSITIVITY OF HUMAN STRAINS OF *M. tuberculosis* ISOLATED PRIOR TO STREPTOMYCIN TREATMENT

Culture**	Growth on streptomycin oleic acid agar*					Remarks
	0	0.5	1	10	100	
B 1715	Innum. <sup>†</sup>	50	0	0	0	Became resistant
B 5017	Innum.	30	0	0	0	" "
B 5709	Innum.	Num. <sup>‡</sup>	7	0	0	" "
710	Innum.	6	0	0	0	" "
15	Innum.	Num.	0	0	0	" "
H37RV***	Innum.	Num.	0	0	0	" "
B 4240	Innum.	Num.	0	0	0	Remained sensitive
B 2611	Innum.	0	0	0	0	" "
B 6085	Innum.	0	0	0	0	" "
B 5362	Innum.	1	0	0	0	" "
B 1401	Innum.	0	0	0	0	" "
B 5862	Innum.	Num.	75	0	0	" "
B 8886	Num.	30	30	20	0	" "
B 7986	Innum.	0	0	0	0	" "

\* Containing designated amounts of streptomycin expressed in  $\mu\text{gm. per ml. of medium}$ .\*\* Cultures marked B were obtained from Toronto Hospital of Tuberculosis (courtesy of Dr. W. Anderson) where resistance is defined as "growth in concentrations of 50  $\mu\text{gm. of streptomycin per ml. on Herrold's medium}$ " (6).

\*\*\* Included as example of strain capable of developing streptomycin resistance in vitro.

† "Innum." indicates abundant growth with innumerable colonies present.

‡ "Num." indicates presence of from 100 to approximately 500 colonies.

cannot serve as a sure prognostic indication of early development of drug resistance. This is illustrated by the cultures B5862 and B8886, which despite the presence of partially resistant cells, did not develop drug resistance in the course of streptomycin therapy.

Although the initial presence of partially resistant cells may be of significance for the later development of streptomycin resistance, there must be other factors at work which determine the emergence of resistant strains. In this connection, recent clinical reports (3, 4) are of special interest which indicate that streptomycin resistance is encountered predominantly among cases of pulmonary tuberculosis in which, prior to streptomycin treatment, definite caseation or cavitation was present.

The difficulty of predicting, by means of a simple laboratory test, the later development of streptomycin resistance, should not detract from the importance of testing the initial cultures by means of solid media for the presence of streptomycin-resistant cells. In view of the increasing possibility of incoming patients having contracted the infection from carriers of resistant strains, the careful testing of such cultures for their streptomycin sensitivity is a matter of considerable urgency. For such strains of *M. tuberculosis*, even if only partially drug-resistant, may present serious problems which will be the subject of a subsequent communication.

### Acknowledgments

This investigation, which is part of a study on chemotherapy of experimental tuberculosis, was carried out with the financial aid from the National Research Council of Canada.

For the supply of cultures the author is indebted to Dr. W. Anderson, Toronto Hospital for Tuberculosis, Weston, Ont., and to Dr. G. P. Youmans, of The Medical School, Northwestern University, Chicago. Thanks are also due to Miss G. S. Slinn for preparing the oleic acid - albumin agar.

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# THE EFFECTS OF SOME URINARY EXTRACTS ON GASTRIC SECRETION IN THE SHAY OPERATED RAT<sup>1</sup>

By FLOYD R. SKELTON AND GORDON A. GRANT

## Abstract

Following the general procedure of Risley, Raymond, and Barnes the effects of urinary extracts on gastric secretion have been studied in the Shay operated young, male, albino rat. The procedure consisted of ligating the pylorus following a 24 hr. fast. Immediately postoperatively the test material was administered. After a five hour period the stomach was removed, opened, and rinsed into 10 ml. of distilled water. An aliquot of this was titrated for its free and total acid content. Human chorionic gonadotrophin and preparations obtained by the benzoic acid absorption method from human and equine pregnancy urine were studied for their effects on gastric section using the above technique after subcutaneous, intraduodenal, and oral administration. It was found that chorionic gonadotrophin caused gastric secretory depression at a dose level as low as 10 mgm. per rat subcutaneously. Both the human and equine preparations were observed to reduce the gastric secretion proportionately to the dose when administered subcutaneously. When administered by intraduodenal injection at the time of operation, the equine preparation was active but required several times the subcutaneous dose to produce comparable results. Oral administration of both equine and human pregnancy urine extracts showed anti-secretory activity only in the former at the dose levels employed. In addition, the effects of possible complicating factors such as estrogens, pyrogens, and nonspecific damage on gastric secretion have been investigated and found to play no appreciable part in the results produced by the urine fractions.

## Introduction

There is convincing evidence that the intestinal mucosa produces a substance "Enterogastrone" which passes into the circulation and inhibits the secretion of gastric juice (2, 3, 6, 13, 14, 15, 10). A similar effect of various urine extracts on gastric secretion has more recently been reported by many investigators (17, 4, 7, 12). The active substance has been called "Urogastrone" and whether it is a separate entity from enterogastrone is not yet certain (5, 8, 9, 11, 20). The reduction in gastric acidity and in the incidence of peptic ulceration during pregnancy (1, 2, 22) has suggested the possibility of the production of an "anti-secretory" or "anti-ulcer" principle at this time. Consequently studies of pregnancy urine as a possible source of such a substance have been carried on by numerous laboratories. The inhibition of ulceration in the Shay operated rat by extracts of intestine and urine is in general accompanied by a roughly parallel decrease in the volume and acid concentration of the gastric juice. On this basis, Risley, Raymond, and Barnes (18) have recently shown that the measurement of gastric secretory inhibition in the Shay operated rat can be used as an indication of the anti-secretory activity of such extracts. The purpose of this communication is to present additional data on the use of the Shay operated rat in an antisecretory assay and to report the effects of some pregnancy urine preparations as well as some nonspecific procedures on the gastric secretory response.

<sup>1</sup> Manuscript received February 15, 1950.

Contribution from the Research and Biological Laboratories, Ayerst, McKenna, and Harrison, Ltd. Montreal, Canada.

### Materials and Methods

The method employed in this study was essentially the same as that of Risley *et al.* but was adapted to male, albino rats 30 days of age and weighing 50–60 gm. Following a 24 hr. fast the pylorus was ligated under light ether anesthesia and immediately postoperatively the substance to be tested was administered. After an interval of five hours during which time no food or water was allowed, the stomach was removed, opened, and rinsed into 10 ml. of distilled water. The volume of gastric secretion was determined by subtraction and an aliquot was taken for titration with *N*/100 sodium hydroxide to determine the free and total acid of the gastric contents. Finally, the free and total acid in the contents of the stomach was expressed as ml. of *N*/100 hydrochloric acid.

The results of all extracts assayed were evaluated in comparison with simultaneously conducted untreated control groups. Each group consisted of 10 or more animals.

### Results

The experimental observations are presented in the following tables. The expressed values include the standard error of the mean.

TABLE I

#### EFFECT OF SUBCUTANEOUS ADMINISTRATION OF HUMAN PREGNANCY URINE EXTRACTS ON GASTRIC SECRETION IN THE RAT

Treatment	Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of <i>N</i> /100 HCl in gastric contents	
			Free acid	Total acid
Control		2.0 ± 0.27	11.8 ± 1.5	20.3 ± 2.5
Chorionic gonadotrophin	10	1.2 ± 0.31	2.0 ± 0.78	7.6 ± 2.4
Benzoic acid adsorbate of human pregnancy urine	12	0.5 ± 0.07	0	1.8 ± 0.46
	6	0.6 ± 0.12	0	2.8 ± 1.1
	2	0.7 ± 0.11	0.9 ± 0.43	4.3 ± 0.75

Table I illustrates the effects of human pregnancy urine extracts on gastric secretion after subcutaneous injection. A 10 mgm. dose of chorionic gonadotrophin (APL) caused marked reduction in volume and acidity of gastric juice. The benzoic acid adsorbate fraction represents the residual urine extract after the chorionic gonadotrophin has been removed. It can be seen that it was much more active than APL in depressing gastric secretory activity and that there was a general dose-response relationship.

In Table II the results of the subcutaneous administration of a benzoic acid adsorbate from pregnant mare's urine are outlined. This preparation was also highly active and appears to have a similar dose-response relationship.

TABLE II

EFFECT OF SUBCUTANEOUS ADMINISTRATION OF AN EXTRACT FROM PREGNANT MARE'S URINE ON GASTRIC SECRETION IN THE RAT

Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of N/100 HCl in gastric contents	
		Free acid	Total acid
Control	2.2 ± 0.2	15.7 ± 2.0	26.6 ± 2.4
100	0	0	1.9 ± 0.3
50	0.1 ± 0.05	0.2 ± 0.1	3.0 ± 0.4
10	2.1 ± 0.05	13.7 ± 2.2	24.6 ± 1.7

The effect of intraduodenal administration of the benzoic acid adsorbate preparations from both human and equine pregnancy urine has been investigated. Table III shows that following intraduodenal injection the human urine extract caused only slight secretory depression without any apparent dose-response relationship. Table IV shows a progressively increasing secretory inhibition with increasing doses of mare's urine extract. However,

TABLE III

EFFECT OF INTRADUODENAL ADMINISTRATION OF A BENZOIC ACID ADSORBATE OF HUMAN PREGNANCY URINE ON GASTRIC SECRETION IN THE RAT

Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of N/100 HCl in gastric contents	
		Free acid	Total acid
Control	3.2 ± 0.14	24.8 ± 2.0	43.3 ± 2.6
50	2.3 ± 0.2	7.9 ± 2.4	38.8 ± 4.0
30	1.8 ± 0.3	8.0 ± 1.8	20.3 ± 2.6
10	1.8 ± 0.2	10.0 ± 1.8	20.3 ± 2.7

TABLE IV

EFFECT OF INTRADUODENAL ADMINISTRATION OF AN EXTRACT FROM PREGNANT MARE'S URINE ON GASTRIC SECRETION IN THE RAT

Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of N/100 HCl in gastric contents	
		Free acid	Total acid
Control	3.3 ± 0.14	27.4 ± 1.6	40.6 ± 1.8
400	0	0	1.7 ± 0.8
200	0.3 ± 0.09	1.1 ± 0.6	6.1 ± 1.2
100	1.7 ± 0.2	9.8 ± 1.8	20.6 ± 2.3
50	2.6 ± 0.3	19.4 ± 3.6	33.8 ± 4.2

approximately eight times the effective subcutaneous dose of equine urine extract was necessary to obtain an equivalent result.

Oral administration of the urine preparations was effected in the following manner. The extract was given by gavage in equally divided doses, 24 and 16 hr. prior to pyloric ligation to ensure complete absorption. It was found that under these conditions a dose of the human pregnancy urine extract 16 times the effective subcutaneous dose had no effect on gastric secretion (Table V). In contrast a marked depression of volume and acidity of gastric juice was obtained with the equine pregnancy urine extract in a dose only nine times the effective subcutaneous dose (Table VI).

TABLE V

EFFECT OF ORAL ADMINISTRATION OF A HUMAN PREGNANCY URINE EXTRACT ON GASTRIC SECRETION IN THE RAT

Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of N/100 HCl in gastric contents	
		Free acid	Total acid
Control	2.4 ± 0.13	11.0 ± 1.1	23.8 ± 1.6
100	2.3 ± 0.18	12.4 ± 1.6	22.4 ± 1.7

TABLE VI

EFFECT OF ORAL ADMINISTRATION OF AN EQUINE PREGNANCY URINE EXTRACT ON GASTRIC SECRETION IN THE RAT

Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of N/100 HCl in gastric contents	
		Free acid	Total acid
Control	3.4 ± 0.19	27.6 ± 2.5	39.2 ± 2.9
450	1.6 ± 0.16	4.8 ± 0.7	15.1 ± 1.3

To exclude the possibility that some secondary complicating factor would explain the above findings, the effects of the substances and procedures shown in Table VII were investigated. As the urine extracts were not totally estrogen free it was necessary to determine the effects of this hormone on gastric secretion. A dose of sodium estrone sulphate five times greater than the amount present in the largest subcutaneous dose of pregnant mare's urine extract was required to cause any significant depression of gastric secretion and acidity. Consequently it was considered that the estrogen content of the extracts did not explain the previous results. McGinty *et al.* have recently reported some inhibition of ulcer formation in Shay operated rats following intravenous and intraperitoneal injections of bacterial pyrogen (20). Although the mare's urine preparation was substantially free of pyrogens, the human pregnancy urine extract (benzoic acid adsorbate after removal of chorionic

TABLE VII

EFFECTS ON GASTRIC SECRETION OF SOME INTERFERING SUBSTANCES AND NONSPECIFIC PROCEDURES IN THE SHAY OPERATED RAT

Treatment	Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of N/100 HCl in gastric contents	
			Free acid	Total acid
Control		3.2 ± 0.13	30.5 ± 1.9	46.9 ± 2.2
Sodium estrone sulphate	5	1.6 ± 0.17	15.4 ± 2.5	24.5 ± 2.8
Pyrogen*	0.1	3.2 ± 0.05	32.6 ± 2.0	47.8 ± 1.8
Intestinal trauma		1.1 ± 0.13	7.1 ± 1.5	17.9 ± 1.9
10% Formalin	0.1 Ml.	1.7 ± 0.38	14.3 ± 3.5	29.1 ± 6.5
Cervical cord trans- section		0.5 ± 0.29	4.8 ± 1.6	9.3 ± 2.9

\* Pure Microbial Pyrogen. Baxter Laboratories Inc. Morton Grove, Ill.

gonadotrophin) was known to be high in pyrogenic activity. For these reasons it was necessary to investigate the effect of subcutaneous pyrogen administration on gastric secretion under our experimental conditions. It can be seen in the table that at the dose level employed bacterial pyrogens did not show antisecretory activity. The effect of nonspecific stress on gastric secretion was investigated as well, because of the possibility that toxicity of the extracts might explain their effects. While the three types of stress did cause reduction in gastric secretion and acidity, the effect was not comparable in degree to that obtained with the urinary extracts although the animals were obviously more damaged.

### Discussion

The decreased gastric secretion produced by chorionic gonadotrophin (APL) from human pregnancy urine confirms previous reports by Sandweiss (19), Culmer, Atkinson, and Ivy (4), and Broad and Berman (3), who observed a similar effect upon gastric secretion in the dog. The fact that the residual urine fraction is much more potent in antisecretory activity than APL suggests that this action of the gonadotrophin may be due to the presence of anti-secretory substances. This was confirmed by heating the APL, a procedure which destroyed the gonadotrophin activity but not the antisecretory activity.

The extracts from human and equine pregnancy urine with antisecretory effects apparently have fundamental differences. Both are active when administered subcutaneously and both have a rough dose-response relationship. However, when administered intraduodenally and orally the preparation from equine urine had greater antisecretory activity. This is to our knowledge the first report of an orally active antisecretory extract from urine.

The possibility that the estrogen content of our extracts was the causative factor was eliminated. A dose of sodium estrone sulphate larger than the

amount of estrogens in the largest subcutaneous dose of urine extracts produced only slight secretory depression.

Intravenous and intraperitoneal administration of purified bacterial pyrogen in doses of 15-30  $\mu\text{gm}$ . and 100-1000  $\mu\text{gm}$ ., respectively, cause gastric secretory depression as well as a reduction of ulcer incidence in the Shay rats (20). Oral administration was without effect. When administered subcutaneously in our experiments, 100  $\mu\text{gm}$ . of pyrogen had no effect on the secretion of gastric juice.

The depression of gastric secretion due to nonspecific damage is of interest because of the gastric erosions which are known to occur from exposure to stress (21). However, as the stress necessary to produce such an effect was far more than that caused by treatment with the extracts it apparently does not explain our observations.

### Summary and Conclusion

The antisecretory properties of chorionic gonadotrophin (APL) and of preparations obtained by the benzoic acid adsorption method from human and equine pregnancy urine were studied in the Shay operated rat following subcutaneous, intraduodenal, and oral administrations. All three urinary preparations produced gastric secretory depression when administered subcutaneously. Oral treatment with the human and equine urine fractions revealed appreciable depressant effects on gastric secretion and acidity by the equine urine preparation only. When injected intraduodenally at the time of pyloric ligation both human and equine urine extracts were active, the latter showing a definite dose-response relationship.

Bacterial pyrogens were ineffective at the levels employed in altering gastric secretory activity when administered subcutaneously.

Large doses of sodium estrone sulphate as well as severe nonspecific damage caused some reduction of gastric secretion and acidity but insufficient to explain the results obtained with the urinary extracts investigated.

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# A COMPARISON OF STERNAL AND SPINOUS PROCESS MARROW<sup>1</sup>

BY R. A. POLSON AND EVELYN A. PACKHAM

## Abstract

Confirming previous reports, a cytological study of the spinous process and sternal marrow showed a marked similarity in two groups of normal subjects. In each of 12 patients with various hematopoietic disorders samples were taken from the sternum and the spinous process. The marrows from the two sites were nearly identical. These findings suggest that the present knowledge of changes occurring in sternal marrow in disease may be applied to spinous process marrow.

## Introduction

Early in 1948 Huss *et al.* (1) and Loge (2) described a technique for obtaining samples of marrow from the spinous processes of lumbar vertebrae. Their reports suggest that the procedure is easier and more convenient than sternal puncture, and that in subjects without gross abnormalities of the marrow, samples from the two sites are essentially similar. The purpose of the present report is to confirm these findings and to show that a close resemblance exists between marrow from these two sites even in conditions characterized by wide deviations from the normal.

## Methods

The sites chosen for marrow aspiration were the sternum and the spinous process of the second or third lumbar vertebra. All sternal punctures were performed using a straight Osgood needle.

Spinous process punctures were performed by the method described by Loge (2). The patient either lay on his side or sat with his back flexed. After preparation of the area with iodine and alcohol, the skin, subcutaneous tissues, and periosteum over the selected spinous process were infiltrated with 2% novocaine. A straight Osgood marrow puncture needle was then introduced directly over the spinous process. The cortical bone was perforated with a rotatory motion of the needle and the narrow cavity entered. A well-fitting dry syringe was attached to the needle and 0.5 ml. of marrow were withdrawn by a brisk pull on the plunger. This maneuver seemed to reduce the admixture of blood in the marrow specimen. Differential cell counts on 500 cells were done on each specimen and total nucleated cell counts per cubic millimeter were performed in most instances.

## Results

Sternal marrow aspirations were performed on one group of 11 healthy laboratory technicians and internes, and spinous process aspirations on

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Contribution from the Departments of Medicine and Pathology of The University of Manitoba and The Winnipeg General Hospital, Winnipeg, Canada.

another group of 12 similar subjects. The results are summarized in Table I, which shows the range and mean values for each type of cell. It will be seen that the marrows from the two sites showed a marked similarity.

TABLE I

## DIFFERENTIAL CELL COUNTS OF THE STERNAL AND SPINOUS PROCESS MARROW OF NORMAL SUBJECTS

Cell type	Spine, 12 subjects		Sternum, 11 subjects	
	Mean	Range	Mean	Range
Blasts	0.8	0.4-1.6	0.4	0.2-1.0
Promyelocytes	3.9	2.2-6.6	3.4	2.6-4.8
Myelocytes	6.8	4.0-9.6	7.3	3.2-8.6
Metamyelocytes	6.7	5.0-11.8	6.8	3.8-9.0
Polymorphonuclears, young	20.3	12.6-27.8	18.1	13.0-22.0
Polymorphonuclears, mature	23.8	18.6-30.4	19.4	13.6-22.6
Eosinophiles	3.4	1.0-5.0	3.2	1.2-5.4
Basophiles	0.4	0-1.0	0.3	0-1.2
Plasma cells	0.3	0-1.2	0.1	0-0.8
Disintegrated	7.6	3.0-15.0	8.5	4.4-11.6
Unclassified	0	0-0.4	0	0-0.2
Lymphocytes	12.1	6.6-22.0	14.5	10.0-23.0
Lymphocytes, immature	0	0-0	0	0-0
Monocytes	2.8	1.0-4.6	2.5	1.0-3.8
Monocytes, immature	0	0-0	0	0-0
Megaloblasts	0	0-0.2	0.1	0-0.6
Erythroblasts	4.6	0.8-9.0	6.0	3.4-8.2
Normoblasts	6.7	2.0-13.4	7.2	6.0-14.6
Nucleated cells per cu. mm. marrow (in thousands)	45	19-75	45	18-100

Simultaneous sternal and spinous process punctures were performed on 13 patients with various hemopoietic diseases. The results are shown in Tables II and III. In Table II it will be seen that in six patients with lymphocytic leukemia the pathognomonic increase in the lymphocytic series was found in both types of marrow, and the degree of abnormality showed a striking correspondence. A similar correspondence was found in the counts of the granulocytic series of cells in the two cases of myelocytic leukemia reported in Table III. In the four cases of secondary anemia also reported in Table III it will be seen that moderately large variations were encountered, particularly in the counts of the erythroblasts. However, the agreement is as close as that found by Reich and Kolb (3), who compared marrows taken from different parts of the sternum. In one case of pernicious anemia, the cell counts of the sternal and spinous process marrow showed a marked similarity in the increase in megaloblasts which characterize this disease.

Each patient who underwent both sternal and spinous process marrow puncture was questioned as to his preference. In the great majority of cases, the spinous process procedure was preferred. In a small number no difference in discomfort was noted, and in no case was sternal puncture preferred.

TABLE II  
DIFFERENTIAL CELL COUNTS OF THE STERNAL AND SPINOUS PROCESS MARROW OF PATIENTS WITH LYMPHOCYTIC LEUKEMIA

Cell type	Source						Case No.
	1	2	3	4	5	6	
SP*	SP**	SP	ST	SP	ST	SP	ST
Blasts	0.2	0.2	0	0	0.2	0.6	0.8
Promyelocytes	2.6	2.6	0	0.3	3.0	3.8	3.0
Myelocytes	3.2	3.2	0	0	4.6	6.8	4.6
Metamyelocytes	2.6	2.6	0.2	0.4	3.2	3.5	4.0
Polymorphonuclears, young	2.2	9.0	1.4	0.4	4.0	10.0	13.6
Polymorphonuclears, mature	13.0	13.0	2.8	1.6	3.6	10.8	10.0
Eosinophiles	0.8	0.4	0	0.4	0	0.2	1.8
Basophiles	0.4	0.6	0.2	0	0	0.4	0.2
Plasma cells	0.2	0.4	0	0	0.3	0	0.2
Disintegrated	12.8	10.4	19.4	19.0	7.6	12.6	15.2
Unclassified	0	0	0	0	0	1.0	3.3
Lymphocytes	45.0	41.0	70.0	71.6	83.3	78.0	34.0
Lymphocytes, immature	8.6	3.0	5.6	5.0	0.3	0.6	5.0
Monocytes	11.4	4.6	0.2	0.2	0.3	0.3	2.0
Monocytes, immature	0	0	0	0	0	0	1.0
Megaloblasts	0	0	0	0	0	0	0
Erythroblasts	0	4.0	0.2	0.6	0	2.0	2.0
Normoblasts	0.6	6.0	1.0	0.8	0	3.6	5.2
Nucleated cells per cu. mm. (in thousands)	27	64	121	267	196	200	95
					26	75	58
							126

\* SP—Spinous process marrow.

\*\* ST—Sternal marrow.

TABLE III  
DIFFERENTIAL CELL COUNTS OF THE STERNAL AND SPINOUS PROCESS MARROW OF PATIENTS WITH MYELOCYTIC LEUKEMIA, SECONDARY ANEMIA, AND  
PERNICIOUS ANEMIA

Cell type	Myelocytic leukemia						Secondary anemia						Pernicious anemia	
	1	2	1	2	3	4	1	2	3	4	5	6	1	1
	Source												Case No.	
	SP*	ST**	SP	ST	SP	ST	SP	ST	SP	ST	SP	ST	SP	ST
Blasts	1.4	1.6	2.6	2.4	0.6	0.8	0.8	0.8	0.6	1.0	1.6	0.8	1.8	2.2
Promyelocytes	12.0	14.8	3.4	3.4	3.6	6.8	2.2	6.8	3.4	5.0	7.2	3.0	6.8	6.6
Myelocytes	11.8	13.8	3.8	3.0	5.6	8.2	6.6	7.2	2.6	5.5	8.0	7.4	6.0	4.0
Metamyelocytes	10.4	8.0	11.4	9.4	2.8	4.8	4.4	4.0	6.6	10.6	9.2	7.0	4.2	4.2
Polymorphonuclears, young	26.6	28.4	35.6	37.4	17.8	17.0	22.8	22.0	17.6	23.2	23.0	19.0	12.0	11.2
Polymorphonuclears, mature	20.2	19.0	21.2	23.4	21.8	13.2	18.2	19.8	29.2	14.2	18.6	24.8	7.0	8.8
Eosinophiles	2.4	2.0	2.8	3.8	3.2	4.0	2.6	3.2	2.4	6.0	6.8	0	2.6	2.8
Basophiles	3.6	3.2	10.0	12.0	0.4	0.2	0.4	0.4	0.2	0.2	0.4	0	0	0
Plasma cells	0	0	0	0.2	0.4	0.6	0.2	0.2	0.6	1.2	1.2	0.4	0	0.4
Disintegrated	4.0	4.0	3.0	1.8	15.6	12.0	8.8	9.8	7.4	9.5	5.8	5.0	14.0	13.0
Unclassified	1.0	0	0	0	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0
Lymphocytes	3.8	3.4	0.8	1.2	8.0	6.8	10.0	9.6	15.8	11.0	6.4	7.0	8.2	7.6
Lymphocytes, immature	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Monocytes	1.8	1.2	0.4	0.4	5.2	2.6	2.2	1.0	3.6	3.2	2.0	1.4	0	1.0
Monocytes, immature	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Megaloblasts	0	0	0	0	0	0	0	0	0	0.2	0.4	0.2	10.0	12.4
Erythroblasts	0.6	0.2	1.8	0.4	4.6	9.6	10.0	4.6	6.2	5.7	5.2	12.0	19.0	21.5
Normoblasts	1.2	0.6	3.2	1.2	10.4	4.0	10.8	11.6	6.4	7.0	10.0	11.4	5.6	5.0
(in nucleated cells per cu. mm.)					47	40	60	81	42	70	64			

\* SP—Spinous process marrow.

\*\* ST—Sternal marrow.

### Discussion and Conclusions

The findings in the two groups of normal subjects support the conclusions of Huss *et al.* (1) and Loge (2), that there is a close similarity between marrow from the spinous process and from the sternum.

The results in the 13 abnormal cases show that this similarity also holds for various pathological conditions of the marrow. This suggests that the information which has accumulated concerning sternal marrow variations in disease should be equally applicable to spinous process marrow.

Since spinous process puncture is as easy, as safe, and gives the same information as sternal puncture while causing less discomfort to the patient, it is suggested that this is the procedure of choice.

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## THE EFFECT OF NIACIN ON THE PYRIDINE NUCLEOTIDE CONTENT OF HUMAN BLOOD CELLS IN ANEMIA<sup>1</sup>

BY M. C. BLANCHAER, D. E. BERGSAGEL, PAMELA WEISS, AND  
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### Abstract

The pyridine nucleotide content of the blood cells (PN/BC) of four normal individuals and five anemic patients was measured repeatedly before and after supplementing the diet with niacin. Daily doses of 50 mgm. nicotinic acid or nicotinamide failed to affect the values in the normal subjects and two well-nourished anemic patients. The same dose of nicotinic acid rapidly raised the PN/BC of three poorly nourished anemic patients to values approximating those of well-nourished subjects with similar degrees of anemia. The results obtained after saturation with niacin confirmed the previous report that the PN/BC is higher in anemic patients than in normal controls and that a negative correlation exists between the pyridine nucleotide values and the severity of the anemia. In spite of continued niacin therapy, correction of the anemia was accompanied by a gradual decrease in the values until they approached those of the normal subjects. The present findings also confirm the earlier report that changes in the PN/BC are apparently independent of moderate variations in the number of circulating leucocytes and reticulocytes and bear no relationship to the size or hemoglobin content of the red cells. The significance of these findings in relation to human niacin nutrition is discussed.

### Introduction

In a recent study (2) it was shown that the pyridine nucleotide content of the blood cells (PN/BC) of well-nourished but anemic patients is higher than that of normal subjects. A negative correlation was observed between the PN/BC and the severity of the anemia when the latter was expressed as the logarithm of either the red cell count or the hemoglobin concentration. Since the PN/BC of poorly nourished patients was distinctly lower than that of well nourished subjects with a similar degree of anemia, it was felt that such measurements might prove of value in the assessment of human nutrition.

The present study deals with the effect of niacin therapy on the PN/BC of normal subjects and anemic patients in various states of nutritional sufficiency. The influence of changes in the red cell count, hemoglobin concentration, and leucocyte count on the PN/BC was also investigated.

### Methods

The subjects were four apparently healthy laboratory workers and five patients suffering from various types of primary and secondary anemia. Clinical data pertaining to the nutritional status of each patient may be found in the section dealing with the results.

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Contribution from the Nutrition Laboratory of the Department of Physiology and Medical Research, The University of Manitoba, and from The Winnipeg General Hospital, Winnipeg, Canada.

The patients were investigated in the following manner. Hematologic and PN/BC measurements were made before and after supplementing an adequate diet, offered *ad libitum*, with niacin. The daily niacin supplement in most cases consisted of 50 mgm. nicotinamide or nicotinic acid. One patient received instead 10 mgm. nicotinamide daily in the form of a multiple vitamin capsule ("Supplavite"). Depending on clinical expediency, specific treatment of the anemia was begun before or after the patients had been placed on the increased level of niacin intake.

A similar plan was adopted in the investigation of the normal laboratory workers, except that these subjects were maintained for longer periods on the various levels of niacin intake.

Details of the hematological and chemical methods used in this work have been described (2). The fluorometric procedure (6) used in estimating the PN/BC does not distinguish between diphosphopyridine nucleotide and triphosphopyridine nucleotide. The blood cell PN content calculated as diphosphopyridine nucleotide was expressed in micrograms per milliliter of cells (PN/ml.), micrograms per gram of hemoglobin (PN/Hb), and micrograms per billion ( $10^9$ ) red cells (PN/RBC).

### Results

The effect of niacin therapy on the PN/BC of four apparently healthy laboratory workers is shown in Table I. Supplementing the diet of these subjects with a vitamin capsule or 50 mgm. nicotinic acid or nicotinamide daily resulted in minor fluctuations in the cellular PN content. These changes were similar in magnitude to the spontaneous variations reported by previous workers (5). The values at no time approached the high PN/BC found in well-nourished anemic patients (2). Although no comparable data is available in the literature on the effect of niacin in physiological amounts on the PN/BC, it is of interest that large doses of nicotinic acid (20 mgm. per kilo per day) produce a rapid elevation of the PN/BC (1, 4) while similar doses of nicotinamide have no effect (3).

The effect of adding 10 mgm. nicotinamide daily to the diet of a well-nourished woman of 35 years with a primary iron deficiency anemia is shown in Fig. 1A. During the first 17 days of the investigation, a decrease in the red cell count and hemoglobin concentration occurred which was accompanied by a small rise in the PN/RBC and PN/Hb values. Iron therapy produced a prompt improvement in the anemia. However, in spite of continued niacin therapy, the PN/BC decreased as the hemoglobin concentration and red cell count rose. The changes in the PN levels appeared to be unrelated to variations in the leucocyte count.

The second patient studied was a woman of 75 years with severe pernicious anemia. No neurological or gastrointestinal disturbances were present. No weight loss had occurred although there was a history of recent anorexia. The effect of nicotinic acid and parenteral liver extract therapy on the blood cell PN levels of this subject is shown in Fig. 1B. Administration of nicotinic

TABLE I  
THE EFFECT OF NIACIN THERAPY ON THE PYRIDINE NUCLEOTIDE CONTENT OF BLOOD CELLS OF NORMAL SUBJECTS

Subject	Day	W.B.C. per cu.mm.	R.B.C. millions per cu.mm.	Hemo- globin, gm.%	Hemato- crit	M.C.V.* $\mu$ l.	M.C.H.C.** %	PN/BC			Niacin therapy, mgm. per day
								PN/ml.***	PN/RBC†	PN/Hb††	
1	0	7400	6.4	16.4	48.5	78	34	72	5.5	210	Basal
	21	—	5.7	15.8	47.5	82	33	73	6.0	220	Nicotinamide, 10 mgm., 20 days
	36	4560	5.0	15.2	48.2	94	32	75	7.3	240	Nicotinic acid, 50 mgm., 14 days
	55	—	—	15.4	47.4	—	—	60	—	180	Nicotinamide, 50 mgm., 18 days
	160	8280	5.1	15.9	51.5	101	31	66	6.7	210 *	Basal for 104 days
	2	0	3600	4.9	13.4	42.6	86	32	65	5.6	210
2	18	—	4.5	13.2	41.9	92	32	60	5.6	190	Nicotinamide, 10 mgm., 16 days
	43	—	—	14.7	44.5	—	—	57	—	170	Nicotinic acid, 50 mgm., 14 days
	3	0	5500	5.3	14.8	50.5	96	28	64	6.1	220
4	14	7100	6.1	15.5	50.5	83	31	74	6.1	240	Nicotinic acid, 50 mgm., 14 days
	0	5600	4.2	13.8	45.5	108	30	58	6.2	190	Basal
	20	8550	6.0	14.4	48.0	81	30	70	5.7	240	Nicotinic acid, 50 mgm., 19 days
	41	9225	6.7	15.5	49.5	74	31	64	4.7	200	Nicotinamide, 50 mgm., 19 days

\* Mean corpuscular volume (red cells).

\*\* Mean corpuscular hemoglobin concentration.

\*\*\* Micrograms pyridine nucleotide per milliliter blood cells.

† Micrograms pyridine nucleotide per  $10^6$  red cells.

†† Micrograms pyridine nucleotide per gram hemoglobin.

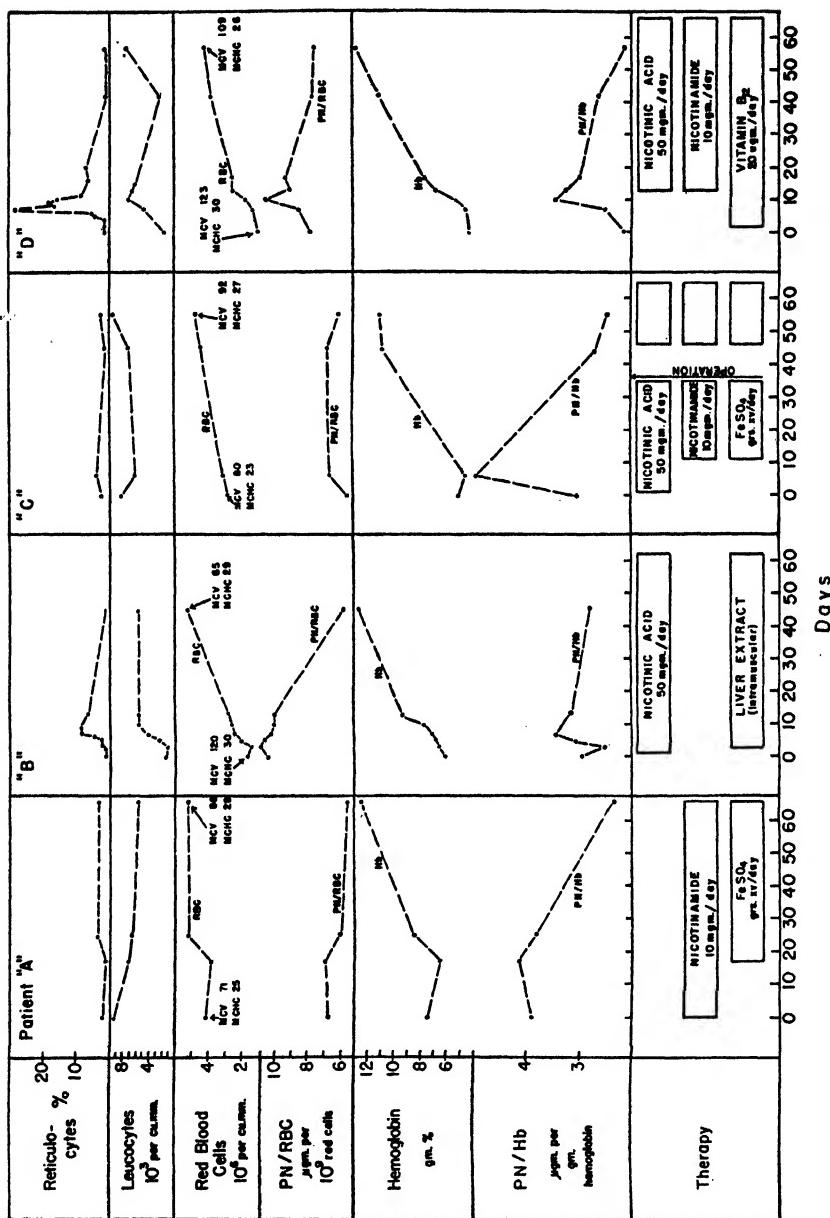


FIG. 1. Effect of therapy on the blood cells and their pyridine nucleotide content in anemia.

acid on the first and second day of the study resulted in a small increase in the PN/RBC and a decrease in the PN/Hb values. Intramuscular injections of liver extract produced a moderate reticulocyte response and a rapid rise in the red cell count, hemoglobin concentration, and leucocyte count. It will be seen that the appearance of increasing numbers of reticulocytes beginning on the fourth day was accompanied by a fall in the PN/RBC values. This observation eliminated the possibility that the simultaneous rise in the PN/Hb was entirely due to the increased number of circulating immature and young red cells and casts doubt on the suggestion of Kohn and Bernheim (5) that the elevated PN/BC of a case of pernicious anemia in their series was due to the presence of reticulocytes. The changes in the PN/BC were not well correlated with the characteristic response of the leucocyte count in pernicious anemia to liver therapy. In spite of a continued elevated nicotinic acid intake, the PN/Hb and PN/RBC values decreased as the hemoglobin concentration and red cell count rose until the cellular PN content approached that of the normal subjects in Table I.

The remaining three patients studied were poorly nourished. The first of these was a man of 59 years with a severe microcytic hypochromic anemia due to chronic bleeding. This subject habitually consumed an inadequate diet and reported recent anorexia and weight loss. No overt physical signs of vitamin deficiency were present. The loss of blood and the subject's poor nutritional status was traced to a partial obstruction of the stoma of a gastrojejunostomy by a phytobezoar. The effect on the PN/BC of nicotinic acid and iron therapy followed by operative removal of the gastric obstruction is shown in Fig. 1C. Administration of nicotinic acid alone resulted in a marked rise in the PN/RBC and PN/Hb values by the seventh day of the study. Eleven days postoperatively, the red cell count had risen to 4.3 millions per cu.mm. and the hemoglobin concentration to 10.9 gm.%. The total plasma protein concentration rose during the course of treatment from 4.6 to 7.9 gm.%. This improvement in the anemia, as in the previous patients, was associated with a drop in the blood cell PN content. It seems unlikely that the decrease in the cellular PN levels was due to a deleterious effect of the operation on the subject's nutritional status since resumption of intensive niacin therapy produced no rise in the PN values. No correlation was observed between the changes in the blood cell PN content and the leucocyte count.

The fourth patient investigated was a man of 65 years with scurvy, massive edema, steatorrhoea, and a severe macrocytic anemia associated with non-tropical sprue. On admission to hospital the plasma protein concentration was 4.1 gm.%. No physical signs of niacin deficiency were found. The data on this subject are shown in Fig. 1D. Parenteral vitamin B<sub>12</sub> therapy produced a rapid rise in the number of circulating leucocytes and reticulocytes which coincided with a moderate increase in the PN/BC. Although the latter rose still further on niacin therapy, the PN values subsequently decreased as the hemoglobin concentration and red cell count increased.

TABLE II  
THE EFFECT OF NIACIN THERAPY ON THE PYRIDINE NUCLEOTIDE CONTENT OF THE BLOOD CELLS IN A CASE OF PERNICIOUS ANEMIA  
*Patient E*

Day	W.B.C. per cu.mm.	R.B.C. millions per cu.mm.	Hemo- globin, gm.%	Hemato- crit	Reticulo- cytes, %	M.C.V.* $\mu\mu l$ .	M.C.H.C.** %	PN/BC			Niacin therapy, mgm. per day
								PN/ml.***	PN/RBC†	PN/Hb††	
0	3800	1.3	4.6	15.4	0.9	119	30	74	8.8	250	Basal
3	2700	1.2	4.8	14.1	0.7	117	34	106	12.3	310	Nicotinic acid, 50 mgm. 2 days
6	4800	1.0	4.4	14.3	0.4	143	31	102	14.5	330	Nicotinic acid, 50 mgm. 5 days

\* Mean corpuscular volume (red cells).

\*\* Mean corpuscular hemoglobin concentration.

\*\*\* Micrograms pyridine nucleotide per milliliter blood cells.

† Micrograms pyridine nucleotide per  $10^6$  red cells.

†† Micrograms pyridine nucleotide per gram hemoglobin.

Since these results suggested that the initial increase in the PN/BC of poorly nourished patients with macrocytic anemia is dependent upon a simultaneous rise in the leucocyte and reticulocyte counts, a woman of 65 years with untreated pernicious anemia was studied. The diet had been low in vegetables, fresh fruits, and animal protein for some time prior to investigation. The results are presented in Table II. Addition of 50 mgm. nicotinic acid daily to the diet was followed by a marked rise in the PN/Hb and PN/RBC values in the absence of any significant change in the red cell count, hemoglobin concentration, leucocyte and reticulocyte counts.

Previous work (2) has indicated that a negative correlation exists between the PN/Hb values of well nourished subjects and the logarithm of the hemoglobin concentration. It was also found that the PN/Hb levels of poorly

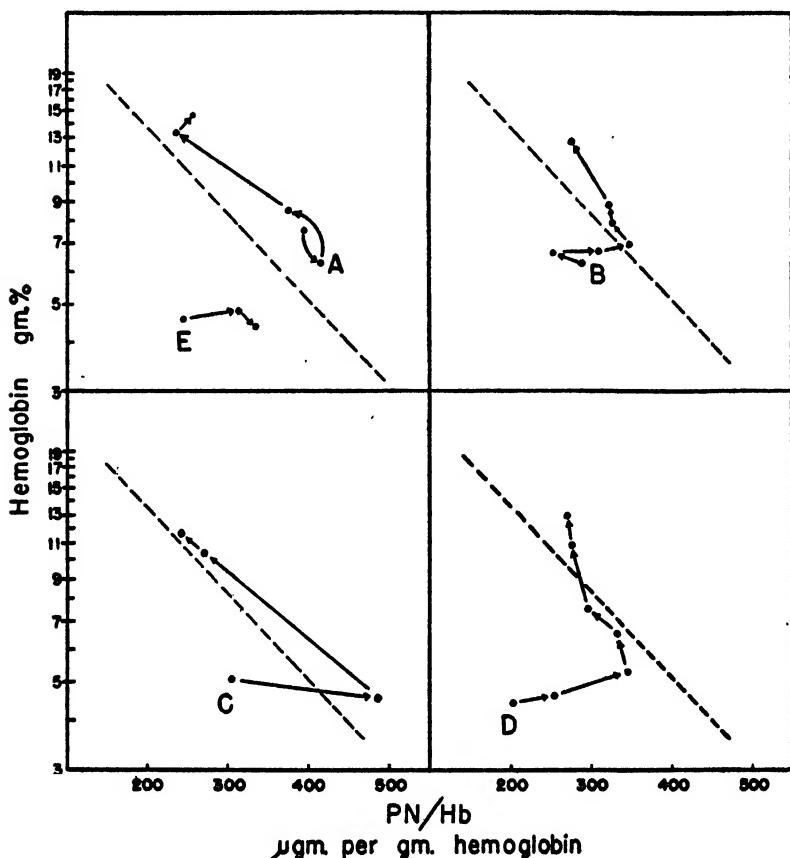


FIG. 2. Data from Fig. 1 and Table II plotted to show the relationship of  $PN/Hb$  to hemoglobin concentration.

nourished patients were lower than those of well nourished subjects with similar degrees of anemia. In order to re-examine this relationship, the corresponding data on the five patients in the present study were plotted in Fig. 2.

For purposes of comparison, the approximate regression line of the relationship previously found (2) between these variables in well-nourished subjects has been included in the figure.

It may be seen that the basal PN/Hb value of each patient, with the exception of the first, was lower than the mean value for well nourished subjects with comparable hemoglobin concentrations. In each case there was good agreement between the patient's general nutritional status and the distance of the initial PN/Hb value from the regression line. With the exception of the first patient, the PN/Hb values rose following niacin therapy and tended to approach the "normal" reference line. As the anemia responded to treatment, the PN levels decreased in spite of a continued elevated niacin intake.

### Discussion

In presenting the results of this study, an attempt has been made to differentiate between the effect of supplementary niacin on the PN/BC and the changes which these values undergo during the response of anemias to specific therapy. It was found that in anemic patients with no evidence of malnutrition, supplementing the diet with niacin had little effect on the PN/BC, while in malnourished patients supplementation raised the values. Correction of the anemia after the values had been raised to "normal" was followed by a gradual decrease in the cellular PN levels until they approached those of the healthy controls. The latter observation confirms the negative correlation between the severity of the anemia and the PN/BC previously described (2) in a group of well nourished subjects with varying degrees of anemia. The present findings also confirm the earlier report that changes in the PN/BC are apparently independent of moderate variations in the number of circulating leucocytes and reticulocytes and that the changes occur in both macrocytic and microcytic types of anemia.

Since none of the patients referred to in this or the previous report showed overt clinical signs of pellagra, no evidence is available to indicate that malnourished subjects are deleteriously affected by low PN/BC levels. However, it is clear that the low values of patients with signs of general malnutrition can be raised by niacin therapy to the levels found in well nourished subjects with comparable degrees of anemia and that the PN/BC in well nourished subjects cannot be raised further by niacin therapy. While such findings suggest that low PN/BC values reflect a state of niacin deficiency, it is felt that this interpretation of the results is acceptable only if it can be shown that the lowered values are associated with a disturbed metabolic activity of the blood cells or can be correlated with some other sign of deficiency. Should further work provide such evidence, it appears that PN/BC values, taking into consideration the effect of anemia, will provide a sensitive index of niacin nutrition.

### Acknowledgments

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## ON RAT SERUM LIPASE

### 1. A SIMPLE MICROMETHOD FOR ESTIMATION OF LIPASE ACTIVITY IN RAT SERUM<sup>1</sup>

BY JULES TUBA AND ROBERT HOARE

#### **Abstract**

A titrimetric micromethod is described for estimating the lipolytic action of rat serum on tributyrin, tripropionin, and ethyl butyrate. A study has been made of factors affecting the action of the enzyme. Values for serum lipase levels of normal adult male and female rats are presented for the three substrates.

#### **Introduction**

A variety of names is given in the literature to the enzyme found in the serum of rats which is capable of hydrolyzing the lower molecular weight triglyceride fats and the fatty acid esters of monatomic alcohols. It is proposed to refer to the enzyme system which hydrolyzes tributyrin, tripropionin, and ethyl butyrate as lipase and its action as lipolysis.

The study of the lipolytic activity of rat serum under various experimental conditions necessitated the development of a suitable and simple microtechnique. In addition, it was essential to obtain the normal range of activity of the enzyme for each of the above substrates.

#### **Experimental**

Adult albino rats, Wistar strain, were used for the investigations reported here. They were fed Purina Fox Checkers and tap water ad libitum.

Blood for lipase determinations was always taken at 8.00 a.m. When the lipase activity of an individual animal was followed at various time intervals, sufficient tail blood was taken to yield at least 0.2 ml. serum, the amount required for a control and an experimental determination. Larger amounts of serum, required for studies of kinetics, were obtained by decapitation and pooling of the blood from several animals. Lipase concentrations were determined usually within 24 hr. after the serum was obtained, although the enzyme level has been found to be unchanged up to two weeks if stored at 5° C. without preservatives.

#### *Factors Affecting the Action of Lipase*

It was decided to avoid the use of emulsifying agents, such as bile or bile salts, in order to maintain as simple a procedure as possible. It was found that mechanical agitation of the enzyme-buffer-substrate mixture during the

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Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta., with financial assistance from the National Research Council, Ottawa, Canada.

digestion period was sufficient to maintain the substrate in a state of fine dispersion, and the use of an emulsifier did not increase the amount of hydrolysis. Goldstein, Epstein, and Roe (2) report very satisfactory lipase estimations in a macromethod where they dispense with an emulsifying agent and use a hand homogenizer to emulsify the substrate and buffer. An important factor in the choice of a buffer is its ability to prevent a pH drift from the optimum due to acid production during the digestion period. Most satisfactory in this regard are sodium diethyl barbiturate - hydrochloric acid buffers, covering the pH range 6.8 to 8.6.

The influence of pH upon the activity of lipase for the three substrates used in the investigations is shown in Fig. 1. The pH optima are; 8.05 for tributyrin, 7.6 for tripropionin, and 7.2 for ethyl butyrate; and the respective decreases in pH during hydrolysis are 0.15, 0.35, and 0.25 units. The

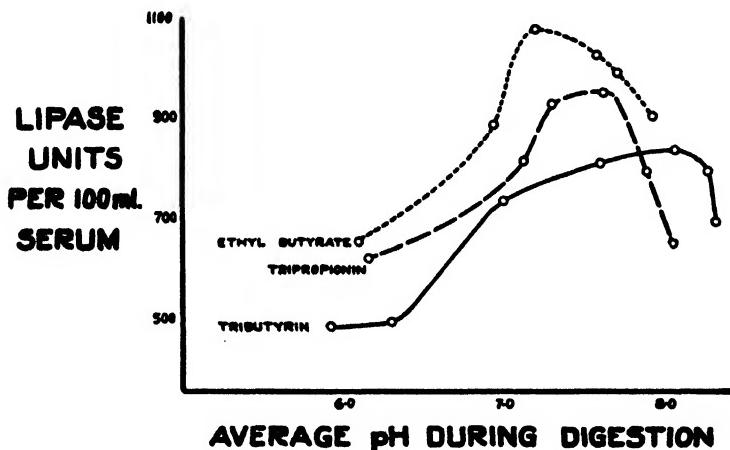


FIG. 1. Effect of pH on hydrolysis of tributyrin, tripropionin, and ethyl butyrate by rat serum lipase.

amount of the veronal buffer required in a digestion mixture varied with the substrate used: 1.0 ml. for tributyrin; 2.0 ml. for tripropionin; and 3.0 ml. for ethyl butyrate. (See procedure below.)

The time interval chosen for enzyme lipolysis was kept within the linear part of the time-activity curve. This minimized the pH drift away from the optimum. Accordingly the most suitable period for hydrolysis was found to be 30 min. for tributyrin, 15 min. for tripropionin, and 30 min. for ethyl butyrate. These intervals were long enough in each case to set free sufficient acid to be accurately estimated.

The relationship between concentration of substrate and enzyme activity is illustrated in Fig. 2 for each of the three substrates. It was decided to use 0.02 ml. tributyrin; 0.025 ml. tripropionin; and 0.015 ml. ethyl butyrate. (See the procedure below.)

After consideration of the several methods described by various workers for estimating the amount of fatty acid set free during lipolysis, a simple titrimetric method was adopted with sodium hydroxide, using phenolphthalein as an indicator.

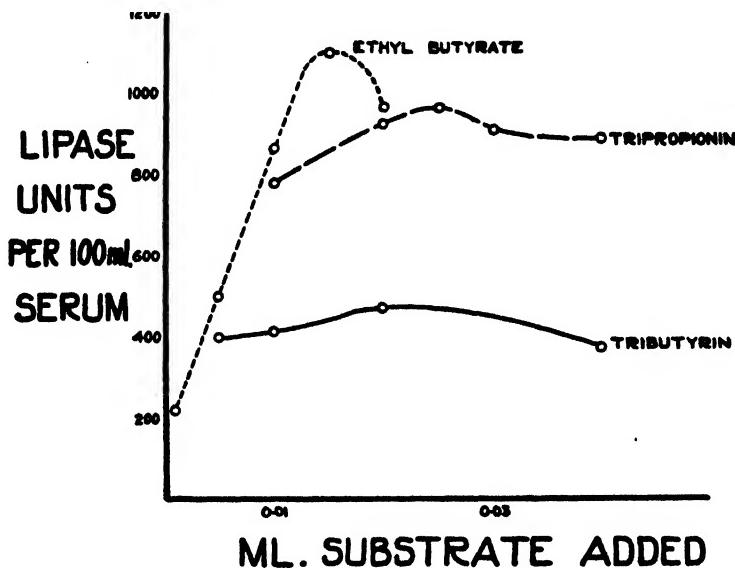


FIG. 2. Effect of substrate concentration on hydrolysis of tributyrin, tripropionin, and ethyl butyrate by rat serum lipase.

### *Procedure for Microdetermination of Serum Lipase Activity*

#### Reagents

Substrates: tributyrin, tripropionin, ethyl butyrate.

Buffers: 0.1 M sodium diethyl barbiturate adjusted with hydrochloric acid to pH 8.2 for tributyrin, pH 7.8 for tripropionin, and pH 7.4 for ethyl butyrate.

Lipase inactivator: 95% ethyl alcohol.

Sodium hydroxide: 0.025 N.

The method for tributyrin will be described in detail and any necessary modifications for tripropionin (P) and ethyl butyrate (EB) will be indicated in brackets.

Micro-Kjeldahl tubes of approximately 5 ml. capacity are used for estimating enzyme activity. Into one of these tubes are pipetted 0.1 ml. serum, 0.2 ml. water, and 1.0 ml. buffer of pH 8.2 (P = 2.0 ml. buffer pH 7.8; EB = 3.0 ml. buffer pH 7.4). The mixture is warmed to 37° C., and then 0.020 ml. tributyrin (P = 0.025 ml.; EB = 0.015 ml.), previously warmed to 37° C., is added with rapid shaking. The dilution of serum in digestion mixtures containing tributyrin, tripropionin, and ethyl butyrate is 1 : 13, 1 : 23, and 1 : 33, respectively. While a constant total volume for the three substrates

would be advantageous, it was found that the above dilutions were most suitable to obtain optimum buffer action and to keep the digestion mixture at a minimum volume. The same procedure was used in establishing the relationships expressed in Figs. 1 and 2. The contents of the tube are mechanically agitated for 30 min. ( $P = 15$  min.;  $EB = 30$  min.) at  $37^\circ C.$  on a Warburg shaker at a rate of 120 swings a minute, which maintains the substrate in a finely dispersed state. For tributyrin, the pH of the contents of the experimental tube at the end of 30 min. is 7.9, the value reported in an earlier paper (8). However, the average of the initial and the terminal pH values was 8.05 in a number of experiments, and this is considered a better representation of the situation.

Lipase activity is terminated and the serum proteins are precipitated by the addition of 3 ml. 95% ethyl alcohol ( $P = 5$  ml.;  $EB = 8$  ml.). The mixture is centrifuged and the supernatant is poured into a 50 ml. Erlenmeyer flask, which is stoppered at once to minimize the absorption of carbon dioxide. The contents of the flask are titrated with 0.025 *N* sodium hydroxide, using phenolphthalein as an indicator, to a faint but persistent pink color.

A control tube is used, which is identical with the experimental tube except that the serum is boiled before buffer and substrate are added.

Enzyme activity is measured by the difference between the titration values of the experimental and control tubes. The lipase activity of serum in units is equivalent to the number of ml. of 0.025 *N* sodium hydroxide required to neutralize the amount of fatty acid set free by the enzyme contained in 100 ml. serum under the above conditions. One ml. of 0.025 *N* base is equivalent to one lipase unit. Because conditions of hydrolysis are different for each substrate, in all experiments the nature of the substance undergoing hydrolysis should be indicated.

#### *Influence of Enzyme Concentration on Reaction Velocity*

The effect of concentration of the enzyme and its activity with the three substrates is shown in Fig. 3. Varying concentrations of serum are used, while all other substances in the digestion mixtures are present in the quantities indicated above under procedure for microdetermination.

It is apparent from Fig. 3 that the enzyme contained in 0.1 ml. serum (the volume used in routine determinations) is saturated by the optimal concentrations of substrate shown in Fig. 2. The straight line relationship is maintained for at least 50% greater concentration than normal levels of the enzyme in rat sera.

Routine determinations of the lipase activity of serum can be determined most satisfactorily with tributyrin as a substrate. The change from optimum pH during digestion is less than with the other two substrates. The total volume of the digestion mixture plus alcohol, used to terminate enzyme action on this substrate, is a convenient one to handle. The relationship between enzyme concentration and activity permits the saturation by tributyrin of levels of enzyme well above the normal range in rat serum.

Replicates on the same serum, using this relatively simple and rapid micro-method, have repeatedly shown agreement well within the limits of 5% experimental error. The amount of substrate hydrolyzed by normal adult

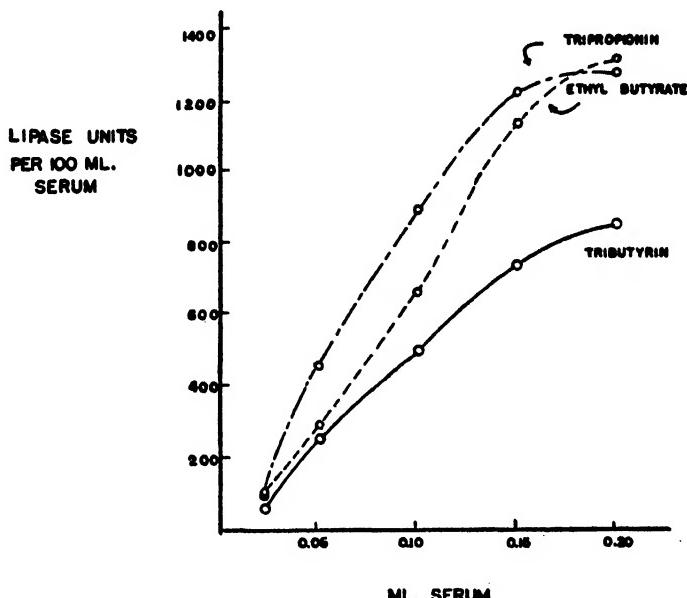


FIG. 3. Relationship between enzyme concentration and lipase activity for tributyrin, tripropionin, and ethyl butyrate.

rat serum lipase is approximately 6% for tributyrin, 7% for tripropionin, and 12% for ethyl butyrate under the above experimental conditions.

#### *Activation and Inhibition*

The accelerating or retarding effect of a limited number of substances on the activity of serum lipase was investigated. Solutions of these substances were pipetted into the experimental tubes in place of the 0.2 ml. water which is usually added, and they were incubated with the serum-buffer mixture for 15 min. before the addition of tributyrin.

The activation of lipase indicated in Table I by calcium chloride is in conformity with the findings of Kraut, Weischer, and Hügel (3), who noted an increase in hydrolysis of pancreatic lipase at pH 8.9 when calcium chloride was added. The same authors report an activation by sodium glycocholate. We were unable to obtain an enhancement of rat serum lipase activity with sodium taurocholate (not included in table), which is in agreement with the findings of Parfentjev, Devrient, and Sokoloff (6) for rabbit serum lipase. An appreciable inhibition was produced with eserine, using a concentration somewhat greater than that of  $10^{-5} M$  which Mendel, Mundell, and Rudney (4) have shown to be sufficient to inhibit completely the cholinesterases of guinea pig plasma. A very marked decrease in lipolysis followed the addition of hexaethyl tetraphosphate. DuBois and Mangun (1) obtained 60%

TABLE I  
EFFECT OF VARIOUS SUBSTANCES ON THE ACTIVITY OF RAT SERUM LIPASE

Substance	Lipase units/100 ml. serum		Effect on lipase activity
	Control	Experimental	
Calcium chloride $3.4 \times 10^{-3} M$	685	850	+24%
Eserine sulphate $2.3 \times 10^{-4} M$	470	290	-38%
Hexaethyl tetraphosphate $1.9 \times 10^{-8} M$	620	85	-86%
Sodium fluoride $7.4 \times 10^{-2} M$	415	40	-91%

inhibition of rat serum cholinesterase using this same inhibitor at a concentration of  $10^{-7} M$ . Almost complete cessation of activity of the enzyme was produced by sodium fluoride. This is an indication, according to Singer (7), that serum lipase is a calcium-requiring enzyme. Evidence in favor of this was found in an additional experiment. The presence of  $7.4 \times 10^{-2} M$  sodium fluoride in an experimental tube lowered the activity of the enzyme 75% (from 620 to 150 units lipase per 100 ml.). In another tube the enzyme was incubated for 30 min. with  $7.4 \times 10^{-2} M$  sodium fluoride, and then  $3.4 \times 10^{-3} M$  calcium chloride was added just before the substrate. The enzyme activity in this case was 705 units per 100 ml. or about 13.5% above the control value.

#### Normal Serum Lipase Values for Adult Rats

These are presented in Table II for the three substrates, tributyrin, tripropionin, and ethyl butyrate. The numbers of animals for the latter two substrates are not large, but they are sufficient to indicate that the rate of

TABLE II  
THE MEAN, THE RANGE, AND THE STANDARD DEVIATION FOR SERUM LIPASE OF NORMAL ADULT RATS FOR TRIBUTYRIN, TRIPROPIONIN, AND ETHYL BUTYRATE

Number of animals	Sex	Substrate	Mean	Range	Standard deviation
56	Male	Tributyrin	596	420-780	$\pm 101$
16	Female	Tributyrin	628	490-840	$\pm 84$
14	Male	Tripropionin	858	720-1050	$\pm 120$
6	Female	Tripropionin	735	630-950	$\pm 104$
16	Male	Ethyl butyrate	734	590-900	$\pm 84$
6	Female	Ethyl butyrate	737	630-900	$\pm 93$

hydrolysis of tripropionin (in 15 min. digestion period) is much greater than for tributyrin, or ethyl butyrate (both with 30 min. digestion periods). Mundell (5) observed that the plasma of mature female rats had greater activity toward acetylcholine than males, due mainly to the nonspecific

cholinesterase. It does not appear from the data of Table II that there is any significant difference in the ability of serum lipase of adult male and female rats to hydrolyze tributyrin.

The ratios of hydrolysis of ethyl butyrate and tributyrin differ in Fig. 2 and Table II. The large volumes of serum required to determine the results expressed in Fig. 2 were obtained by decapitating several animals and pooling their sera. Different pools were used for each substrate. The ratio of hydrolysis for these two substrates indicated in Table II (i.e. above 1.2) is based on sera obtained from tail blood as indicated above. In subsequent experiments on normal adult rats, the ratio has remained constant.

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## SOME PROPERTIES OF NORMAL CHICK ALLANTOIC FLUID IN RELATION TO INFLUENZA VIRUS HEMAGGLUTINATION<sup>1</sup>

By J. R. POLLEY, M. M. BURR, AND A. L. GILLEN

### Abstract

The pH, oxidation-reduction potential, total solids, nonspecific hemagglutination titer, and the titer of the inhibitor of influenza virus hemagglutination have been determined in the normal allantoic fluid of the chick embryo as the incubation time increased. These determinations were repeated after the allantoic fluid had been stored for 48 hr. at 0° C. It was found that as the incubation time increased (1) the pH decreased progressively, (2) the Eh remained positive throughout, (3) the total solid content increased, (4) the nonspecific hemagglutination titer remained negligible, (5) the inhibition titer increased rapidly. Storage for 48 hr. at 0° C. produced no change other than a small rise in the pH. By buffering the allantoic fluid *in vivo*, it appeared that the increasing inhibition titer was not directly related to the decreasing pH. The inhibition titer of the allantoic fluid was not decreased after dialysis, indicating that it is not affected by the amount of urates in the fluid. Thus it appears that the inhibition titer is related directly to a component of the increasing total solids which is unidentified as yet.

At the present time, the embryonated egg is in widespread use for the propagation and passage of numerous viruses. The isolation and identification of many human and animal viruses involves passage through the fertile chicken egg (1, 9), followed by various procedures for the isolation and purification of the viruses from the embryo fluids.

It has been shown that the growth of viruses and their stability are affected by the pH, oxidation-reduction potential, and the temperature of the medium (2, 5, 10, 12). Beard *et al.* (11) found that with mumps virus, the optimal range of pH stability was from 5.8 to 8.0. Influenza A virus (PR8) was most stable at about pH 7 while influenza B virus (Lee) was most stable at pH 7.9 or greater (4). Usually, a neutral or slightly alkaline pH, a reducing medium, and a temperature of about 35°-37° C. favor virus growth.

Changes in the pH and Eh values of the allantoic fluid of normal and influenza infected eggs have been reported (6). With normal allantoic fluid the Eh was usually positive and the pH decreased as the age of the embryo increased. In 1947, Svedmyr (8) showed that normal allantoic fluid contained an inhibitor of influenza virus hemagglutination. Hardy and Horsfall (3) noted that this inhibitor increased with the time of incubation of the eggs and that it was capable of combining with influenza A virus. Following this combination, there was only partial dissociation.

Since a given virus, such as influenza, has an optimal pH for growth and stability, it seems possible that a decreasing pH of allantoic fluid with increasing time of incubation would represent a detriment for continued multiplication. Also, since it has been reported that the titer of the inhibitor of virus hemagglutination increases with time, it seems possible that the low yields of

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virus found beyond certain times of incubation might be due, at least in part, to the fact that the true virus titer was obscured by an increasing inhibitor content. Hence, the present study of normal allantoic fluid was undertaken to follow, not only any changes in the pH and Eh of the fluid but any simultaneous changes which might occur (1) in the titer of the inhibitor of influenza virus hemagglutination and (2) in nonspecific hemagglutination. By giving quantitative expression to these properties determined simultaneously, it was hoped that it might be possible to correlate them with known conditions for optimal virus growth and to demonstrate their effect on virus assays.

### Experimental

White Leghorn eggs received from a single hatchery were used. On arrival, the eggs were weighed individually and only eggs weighing between 55-58 gm. were selected for immediate incubation. The eggs were incubated on their side at  $37.5^{\circ} \pm 0.5^{\circ}$  C. at a relative humidity of 80% in a forced-draught incubator.

The allantoic fluid was harvested under aseptic conditions from viable embryos without rupturing the amniotic sac or the blood vessels of the chorioallantoic membrane. The allantoic fluid from each egg was placed in a separate sterile test tube. The pH, nonspecific hemagglutination, and inhibitor determinations were made immediately on a portion of each specimen. The remaining fluid was placed in an icebox at 0° C. for 48 hr. and then the above determinations were repeated to observe any changes on storage.

The determinations of the pH and the oxidation-reduction potential, Eh, were made using a Beckman model G pH meter. For the pH measurements, micro glass and calomel electrodes supplied with the instrument were used. To determine the Eh, a No. 281 platinum electrode was substituted for the glass electrode in the circuit.

Aliquots of the allantoic fluid samples were tested for the presence of nonspecific hemagglutinins by Salk's method (7).

For the titration of inhibitors of influenza virus hemagglutination in the normal allantoic fluid samples, serial twofold dilutions of known PR8-infected allantoic fluid were prepared in saline. It is important to point out that with these tests to be described, the titer of inhibitor is influenced by the titer of the virus used to determine it (3), hence a common pool of PR8 influenza infected allantoic fluid was used throughout this study. To 0.4 ml. of each dilution of virus was added 0.1 ml. of the allantoic fluid sample under test. The tubes were shaken and 0.5 ml. of a 0.25% suspension in saline of washed chicken cells was added. The tubes were shaken again and kept at room temperature until the cells had settled completely. A control titration of the virus in saline was made at the same time. End points were taken as the last dilution showing complete hemagglutination by Salk's method (7). The results were recorded as the degree of inhibition which is expressed as

the ratio of the virus titer in saline to the titer in the presence of normal allantoic fluid. This is similar to a method used by Hardy and Horsfall (3).

Repeating these tests with heated virus, we were able to confirm the finding that higher inhibition titers were obtained than with unheated virus (3).

Thus, these two titrations give the inhibition value against both heated and unheated virus. Using the heated virus, higher inhibition titers are obtained and there is a wider spread in the values which is desirable for comparing the results for increasing time of incubation. The corresponding values obtained with unheated virus indicate the actual degree of interference to be expected in ordinary virus titrations.

To determine the total solid content of the allantoic fluid, 1 ml. aliquots of each sample were added to tared crucibles and weighed again. The crucibles were placed in an oven at 105° C. for 24 hr. and were then weighed again when cool.

### Results

The results of this study are presented in tabular rather than graphic form to present both the range and the standard deviations of the values with increasing time of incubation. For example, if a graph were plotted between the time of incubation and the mean pH values shown in Table I, the conclusion might be drawn that the decreasing pH was directly related to the

TABLE I  
pH OF NORMAL ALLANTOIC FLUID WITH TIME OF INCUBATION

Time of incubation, days	No. of observations	pH range	Mean pH	After 48 hr. at 0° C.
10	30	7.4 - 8.2	7.9 ± 0.2	8.3 ± 0.3
11	30	7.1 - 8.1	7.7 ± 0.3	8.0 ± 0.3
12	18	7.2 - 8.0	7.5 ± 0.3	8.0 ± 0.3
13	18	6.5 - 7.9	7.3 ± 0.3	7.9 ± 0.3
14	30	6.4 - 7.9	7.0 ± 0.5	7.6 ± 0.5
15	30	5.5 - 7.5	6.5 ± 0.5	6.9 ± 0.5
16	24	5.3 - 7.0	5.8 ± 0.5	6.4 ± 0.6
17	20	5.0 - 6.7	5.6 ± 0.4	6.2 ± 0.5
18	8	5.2 - 6.7	5.6 ± 0.4	6.1 ± 0.5

incubation time. Actually, however, the range of pH values encountered shows that, although the average pH decreases with time, it is impossible to correlate the pH directly with the time of incubation for each individual egg.

From Table I it can be seen that the mean pH decreased with the time of incubation. The decrease was almost linear from 10 to 14 days, then there was a sharper drop to 16 days followed by a levelling off. After being stored for 48 hr. at 0° C., the pH of the allantoic fluid increased by about 0.5.

It can be seen from Table II that the Eh of the samples remained positive throughout the course of this study. Storage at 0° C. had no significant effect on the values.

TABLE II

## Eh OF NORMAL ALLANTOIC FLUID WITH TIME OF INCUBATION

Time of incubation, days	No. of observations	Eh range, mv.	Mean Eh	After 48 hr. at 0° C.
10	20	+ 290 to + 340	+ 310 ± 40	300 ± 50
11	20	+ 290 to + 325	+ 305 ± 40	280 ± 45
12	20	+ 140 to + 280	+ 210 ± 70	250 ± 30
13	20	+ 185 to + 280	+ 245 ± 55	220 ± 45
14	20	+ 265 to + 340	+ 290 ± 35	260 ± 40
15	18	+ 190 to + 300	+ 240 ± 60	260 ± 70
16	20	+ 315 to + 360	+ 330 ± 25	290 ± 60
17	18	+ 285 to + 350	+ 325 ± 40	280 ± 70
18	8	+ 300 to + 360	+ 340 ± 40	300 ± 45

In Table III is shown the weight increase of the embryo, the rate of growth, expressed as gm. per day and the total solid content of the allantoic fluid. There was a gradual increase in the total solid content of the normal allantoic fluid with increasing age of the embryo. The amount, however, was only of the order of from 1 to 1.5 gm. per 100 cc. An indication of a low protein content was given by the fact that the addition of 10% trichloroacetic acid to samples produced only an immediate slight turbidity.

It was found that the titer of nonspecific hemagglutination remained less than 10 units throughout the time of incubation. Also, it did not increase after the fluid had been stored at 0° C. for 48 hr.

From Table IV it can be seen that the titer of the inhibition of influenza hemagglutination increased rapidly with time of incubation. With the heated virus, the inhibition titers are larger than with unheated virus but the range of values is similar in each case, usually being about a fourfold one. For example, at 13 days it is 16 - 64 with heated virus compared with 2 - 8 with unheated virus. Storage at 0° C. for two days did not cause any significant change in the titer, either with heated or unheated virus. The titer of the virus pool used for these tests was 2560 and since the first dilution tube

TABLE III

EMBRYO WEIGHT, RATE OF GROWTH, AND TOTAL SOLID CONTENT OF NORMAL  
ALLANTOIC FLUID WITH INCUBATION TIME

Time of incubation, days	No. of observations	Mean weight, gm.	Growth increment, gm.	Total solids, mgm./gm.
10	30	2.1 ± 0.2	—	8.5 ± 0.5
11	30	2.8 ± 0.2	0.7	9.7 ± 0.5
12	18	4.1 ± 0.3	1.3	9.9 ± 0.5
13	18	5.9 ± 0.3	1.8	10.6 ± 0.4
14	36	7.9 ± 0.4	2.0	10.2 ± 0.5
15	30	11.0 ± 0.4	2.1	10.5 ± 0.6
16	30	13.4 ± 0.5	2.4	12.0 ± 0.7
17	20	16.2 ± 1.0	2.8	15.1 ± 0.8
18	8	20.3 ± 0.4	4.1	15.3 ± 0.7

TABLE IV

TITER OF INHIBITOR OF INFLUENZA VIRUS HEMAGGLUTINATION IN NORMAL  
ALLANTOIC FLUID WITH TIME OF INCUBATION

Time of incubation, days	No. of observations	Inhibition titer			
		Immediate		After 48 hr. at 0° C.	
		Heated virus	Unheated virus	Heated virus	Unheated virus
10	30	4 - 16	2 - 4	8 - 16	2 - 4
11	30	8 - 16	2 - 4	8 - 16	2 - 4
12	18	8 - 32	2 - 8	2 - 16	2 - 8
13	18	16 - 64	2 - 8	8 - 16	2 - 8
14	30	16 - 64	4 - 8	32 - 128	4 - 8
15	30	32 - 128	8 - 32	32 - 128	8 - 16
16	24	128 - 256	8 - 32	128 - 256	8 - 32
17	18	128 - 256	32 - 64	256	32 - 128
18	8	256	64 - 128	256	64 - 128

was 1 : 10, the maximum inhibition titer that could be obtained here was 256. Using heated virus, this was reached on the 16th day of incubation. The inhibition titer with unheated virus was only 8 - 32 at this time but was

rapidly increasing. For practical purposes, an inhibition titer of 8 with unheated virus (which is often reached by the 12th day, the time of routine harvesting of influenza virus) would mean that a virus hemagglutination titer of 2560 would appear as only  $\frac{2560}{8} = 320$  in the presence of normal allantoic fluid.

The following observations, which have been made as the time of incubation (age of embryo) increased, are presented together in an attempt to correlate the data: (1) the pH of the allantoic fluid decreased, (2) the pH increased after storage for two days at 0° C., (3) the oxidation-reduction potential remained positive throughout, (4) the nonspecific hemagglutination titer remained less than 10 units throughout, (5) the total solid content of the allantoic fluid increased, (6) the titer of the inhibitor of influenza virus hemagglutination increased rapidly. Since the oxidation-reduction potential remained positive and without any significant variation and since the non-specific hemagglutination showed no increase, it appears that the increasing inhibition titer may be related to the increasing total solids and/or to the decreasing pH. That it was not the decreased pH per se which caused the rise in inhibition titer was shown by repeating the inhibition titrations in isotonic phosphate buffer, pH 7.0, and obtaining the same values. The fact that the pH of the allantoic fluid rose about 0.5 after storage in the icebox, without significant change in the inhibition titer, suggests that the decreasing pH and the increasing inhibition titer are not directly related. However, this pH change is not sufficiently great to be conclusive.

In an attempt to determine whether there is a direct relationship between the decreasing pH during the time of incubation and the increasing inhibition titer, the allantoic fluid was buffered *in vivo*. The inhibition titers were determined at given time intervals.

In a preliminary experiment, 1.0 ml. of sterile isotonic phosphate buffer, pH 7.5, was injected into the allantoic sac of embryos on the 10th day of incubation. A similar amount of sterile isotonic saline was injected into the allantoic sac of a control group of 10 day embryos. Normal (noninjected) fluids were also tested to serve as normal controls. Allantoic fluids were withdrawn for test on the 14th and 16th day of incubation. It was found that the average pH of the three groups of fluids had fallen to about the same extent. That the buffer had some small effect, however, was indicated by the fact that the buffered allantoic fluids showed less deviation in their pH values from the average value. The inhibition titers were similar in the three groups.

The experiment was repeated as above except that this time 2.0 ml. of 0.1 M phosphate buffer, pH 7.8, was used instead of 1.0 ml. of isotonic phosphate. An equivalent amount of saline was injected into others to serve as treated controls. The results are shown in Table V.

From Table V it can be seen that the action of the buffer is more marked on the 16th than on the 14th day of incubation. On both the 14th and 16th days, the inhibition titers for the three groups lie in similar ranges. There

TABLE V  
pH AND INHIBITION TITER IN NORMAL AND BUFFERED ALLANTOIC FLUID

Treatment	Time of incubation, days	No. of observations	pH	Inhibition titer	Total solids, mgm./gm.
None	14	30	7.0 ± 0.5	16 - 64	10.2
Saline	14	24	6.7 ± 0.6	16 - 64	10.8
Buffer	14	20	7.1 ± 0.5	32 - 64	11.1
None	16	24	5.8 ± 0.5	12 - 256	12.0
Saline	16	20	6.0 ± 0.4	128 - 256	12.5
Buffer	16	20	6.6 ± 0.4	128 - 256	12.8

are differences in the pH values, however. Thus, the pH of normal allantoic fluid fell from 7.0 on the 14th day to 5.8 on the 16th day of incubation, while the buffered fluid decreased only from 7.1 to 6.6. The inhibition titer, however, decreased to the same extent in both normal and buffered fluids. Hence, it appears that the decreasing pH during incubation is not directly related to the increasing titer of the inhibitor of influenza virus hemagglutination.

To determine whether the inhibition titer was associated with the presence of urates in the allantoic fluid, aliquots of a pool of normal allantoic fluid were dialyzed in cellophane bags against 0.85% saline at 0°, 25°, and 37° C. for 48 hr. As controls, aliquots of the allantoic fluid were kept in test tubes at these temperatures. At the end of the dialyzing period, the inhibition titer of all samples was determined. It was found that dialysis for 48 hr. did not alter the inhibition titer at any of the above temperatures. This suggests that the inhibition is not due to the presence of urates in the normal allantoic fluid and that the inhibitor is a compound of relatively large molecular weight.

### Discussion and Summary

One of the most important properties of normal allantoic fluid is its inhibitory action on influenza virus hemagglutination. In the process of harvesting virus from pooled infected allantoic fluids, the inclusion of fluids having little or no virus content decreases the titer of the pool by considerably more than would an equivalent amount of saline. The fact that it has been found that the optimal time for harvesting influenza-infected allantoic fluid is from the 12th - 13th day of incubation may be a reflection of the fact that beyond this time the rapidly increasing inhibitor content masks the true virus content by depressing the experimental titration value.

The increasing concentration of inhibitor in the allantoic fluid was associated with a decreasing pH and an increasing total solid content. By buffering

some allantoic fluid *in vivo* it was shown that the inhibition was not related directly to the decreasing pH. The inhibitory action of allantoic fluid was unchanged after dialysis, indicating that it was not due to the urate fraction of the total solids. Apparently, the increasing inhibition titer is related directly to an increasing component of relatively large molecular weight in the total solids which is unidentified as yet. Further studies are in progress.

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# THE UPTAKE OF RADIOACTIVE PHOSPHORUS BY INFLUENZA VIRUS A (PR8 STRAIN)<sup>1</sup>

By A. F. GRAHAM AND LAURELLA McCLELLAND

## Abstract

Radioactive inorganic phosphorus placed in the allantoic sacs of embryonated eggs three hours after inoculation with influenza virus was incorporated into the structure of the virus during its growth. There was little or no direct exchange between the virus and radioactive inorganic phosphorus. The specific activity of purified labelled virus rose linearly with increasing amounts of radioactive phosphorus administered to the eggs. When radioactive phosphorus was placed in the allantoic sac 48 hr. before inoculation with influenza virus the newly formed labelled virus had a specific activity about 20% higher than when isotope was administered at the same time as virus. As the amount of isotope injected into each infected egg was increased up to 775  $\mu$ rd. an increasing number of embryos died during the subsequent period of virus growth. The yield of virus from the surviving eggs was not less than from eggs which had not received radioactive phosphorus. Under the experimental conditions described the amount of isotope which could be introduced into influenza virus was not sufficient to permit the use of the marked virus in metabolism experiments in animals or embryonated eggs.

## Introduction

The purpose of this work was to determine whether the elementary bodies of influenza virus could be labelled with radioactive phosphorus during their growth in the allantoic membrane of the embryonated egg. If purified influenza virus could be obtained with a sufficiently high content of isotope it was considered that a number of problems in the field of animal viruses would be open to new methods of study.

Thus, in the first place, it would be of interest to determine the fate of the isotope when mouse lung or allantoic membrane was infected with radioactive virus since this might throw some light on the mechanism of cell infection by the virus. Secondly, chemical analysis of radioactive virus would show whether the isotope was concentrated in one or more of the virus constituents, indicating that these constituents played a special role in virus growth. Thirdly, it would be of great interest to compare the rate of uptake of isotope by some of the phosphorus containing constituents of the normal and infected cell; this would give an indication as to whether virus infection alters the phosphorus metabolism of the cell. Fourthly, radioactive influenza virus might be useful in immunological studies along lines suggested by the work of Libby and Madison with labelled tobacco mosaic virus (9).

Some of these problems are general to the study of all viruses, and it appeared that a suitable model system for preliminary study would be that of influenza virus growing in the allantoic membrane. Conditions for growth, methods of purification, and chemical analysis of this virus have already been worked out in detail by previous investigators. Although no published work was available on the application of isotope techniques to the study of animal

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Contribution from Connaught Medical Research Laboratories, University of Toronto, Ont.

viruses, Cohen (1, 2) and Putnam and Kozloff (11) have investigated the growth of  $T_2$ ,  $T_4$ , and  $T_6$  bacteriophages on *Escherichia coli* in the presence of radioactive phosphorus. Since these bacterial viruses readily incorporated the isotope into their structures there was a strong suggestion that influenza virus would also become labelled during its growth in the fertile egg.

The present paper describes the conditions under which radioactive phosphorus was incorporated into the structure of influenza virus. A preliminary report of this work has already appeared (5).

## Methods

### Determination of Total Phosphorus

Total phosphorus was determined by the method of Kuttner and Lichenstein (8) with several modifications introduced to suit the present purpose.

An aliquot of the solution to be estimated was pipetted into a test tube graduated at the 15 ml. mark, and 1.0 ml. of 15 N sulphuric acid was added. The mixture was heated on an open flame until most of the water had been removed and digestion was continued on a sand bath over an electric hot plate for one hour. One drop of concentrated nitric acid was then added and the heating continued a further half hour. One to two ml. of distilled water was added and then evaporated off rapidly over an open flame. This procedure removed decomposition products of nitric acid which interfered seriously with the subsequent color development.

After addition of 2.0 ml. of 5.51% ammonium molybdate (w/v) the volume was made up to about 13 ml. with water and thoroughly mixed. To this was added 1.5 ml. stannous chloride (working solution prepared daily by diluting 1.0 ml. of stock solution, 10 gm. stannous chloride in 25 ml. concentrated hydrochloric acid, to 200 ml. with water) the tube being shaken continuously during addition. The volume was made up to 15 ml., mixed, allowed to stand 10 min. at room temperature, and the per cent transmittance read against distilled water at  $630 \text{ m}\mu$  in a Coleman Spectrophotometer. The calibration curve was linear between 0 to 20  $\mu\text{gm. P}$ .

### Determination of Radioactive Phosphorus ( $P^{32}$ )

Practically all the determinations of radioactivity were made on solutions of material in water, biological fluids, or organic solvents. The method used will be fully described (4).

Throughout this paper the activities of  $P^{32}$  sources are expressed as counts per minute (c.p.m.), that is, the number of impulses registered per minute on a Geiger-Müller counter. Radioactivity measurements were carried out in triplicate for each solution. Each source was measured with a standard deviation of 2 to 5% of the net counting rate (7) unless the net counting rate was less than about 20% of the background, when a standard deviation of 25% was considered to be sufficient accuracy. Since the method was calibrated (4) against a standard RaD + E source from the National Bureau of

Standards, the results may be converted to microrutherfords ( $\mu$ rd.) using the factor 1000 c.p.m. are equivalent to a  $\beta$ -ray activity of 85.8  $\mu$ rd.

The radioactive phosphorus was obtained at monthly intervals from the National Research Council, Chalk River, as phosphoric acid in 0.005 M hydrochloric acid, carrier free. The decay rate of the material was checked experimentally from time to time, but, in general, corrections for decay of  $P^{32}$  during an experiment were calculated from a decay curve assuming a half-life of 14.5 days.

#### *Determination of Protein Nitrogen*

Protein nitrogen was estimated after digestion with sulphuric acid and Perhydrol by the Nessler method using the reagent described by Vanselow (16). The density of the developed brown color was determined at 460 m $\mu$  against the reagent blank. Ammonium sulphate was used as a standard, the calibration curve being linear from 0-60  $\mu$ gm. N.

#### *Determination of Infectivity of Influenza Virus*

Serial tenfold dilutions of virus suspension in broth were injected in 0.2 ml. amounts into 11 day embryonated eggs by the allantoic route. Generally, at least six groups of seven eggs each were used for an infectivity titration. After incubation at 36° C. for 48 hr. a small quantity of allantoic fluid was aspirated from each egg and tested for haemagglutination with a washed suspension of chicken erythrocytes. The 50% infectivity end-point ( $ID_{50}$ ) was calculated according to the method of Reed and Muench (12).

In many cases the virus content of solutions was estimated by the chicken red cell agglutination (CCA) test of Miller and Stanley (10). This test was used only as a rough indication of the amount of virus in a suspension, its reproducibility and accuracy being much inferior to the infectivity titration.

### **Experimental**

#### *Growth, Purification, and Properties of Influenza Virus*

During the course of the work several methods for harvesting and purifying influenza virus were tried but the best preparations were obtained by the following procedure which is similar in many respects to that worked out by Taylor *et al.* (15).

Eleven-day embryonated eggs were inoculated by the allantoic route with 10-100  $ID_{50}$  of influenza virus A (PR8 strain) in 0.2 ml. amount. After incubating the eggs for 48 hr. at 36° C. they were opened under sterile conditions and a large blood vessel of the inner chorioallantoic membrane was severed. The mixture of blood and allantoic fluid was aspirated into 250 ml. centrifuge bottles and allowed to stand at 5° C. for 18 hr. to permit complete agglutination of the red blood cells by the virus. Following centrifugation the agglutinated cells were suspended in ice-cold 0.85% (w/v) sodium chloride adjusted to pH 7.0, hereafter referred to as "saline", and again centrifuged. A volume of saline equivalent to 1/10 the original volume of allantoic fluid was then added and the virus eluted from the cells by incubating at 37° C.

for three hours. The cells were removed by sedimentation in a horizontal centrifuge at 1000 r.p.m. and the supernatant subjected to further centrifugation for 10 min. in an angle centrifuge at 2000 g. The supernatant solution thus obtained was centrifuged at 20,000 g for one hour at 5° C. in a Sorvall angle centrifuge to sediment the virus. A few drops of saline were added to the pellet after the supernatant solution was poured off. Whenever possible the mixture was allowed to stand overnight at 5° C. since the virus resuspended more readily under these conditions. The virus was then resuspended by pipetting for several minutes through a fine-tipped pipette, the volume made up to 1/100 the volume of original allantoic fluid with saline and centrifuged at 5° C. for 10 min. at 2000 g in an angle centrifuge to remove large particles. A second cycle of high and low speed centrifugation was performed on the supernatant solution.

The final virus suspension was a white opalescent liquid estimated by infectivity test to contain 55-70% of the virus in the original allantoic fluid. Such suspensions contained about 2.5 mgm. of virus per ml., calculated from phosphorus analyses, assuming that the virus contained 0.97% P (14).

In some cases the above procedure was modified in that the infected eggs were chilled at 5° C. for 18 hr. before harvesting, and washed chicken erythrocytes were added to the clear allantoic fluid to make a 2% suspension. This method was not as rapid as the one outlined above and suffered the further disadvantage that it was more difficult to maintain sterile conditions. Various media, such as the Ringer - calcium chloride solution described by Taylor *et al.* (15), and phosphate buffer, were used to resuspend the sedimented virus but offered no advantage over 0.85% sodium chloride solution.

Mounts were prepared from several of the purified virus suspensions, fixed in osmic acid vapor, and photographed in the electron microscope before and after shadow casting with chromium.\* In every case the great majority of the particles observed were typical in size and shape of influenza virus with little other electron absorbing material present. In some cases a few of the filamentous forms often observed previously in preparations of this virus were apparent, see for example (13).

Freshly purified suspensions of virus gave a single boundary in the ultracentrifuge, Fig. 1, with a sedimentation constant of about  $670 \times 10^{-13}$  (uncorrected) in agreement with earlier work on the elementary bodies of influenza virus A. With some preparations a slightly raised base line ahead of the main component in sedimentation pictures suggested a small amount of faster moving material, probably clumped virus particles. When purified preparations were allowed to stand several days at 5° C. a small slower moving boundary appeared as described by Friedewald and Pickels (3). The sedimentation velocity measurements were carried out in a Spinco electrically driven ultracentrifuge equipped with a Philpot-Svensson type of optical

\* We are much indebted to Dr. G. D. Scott of the Physics Department, University of Toronto, for making the electron micrographs.

PLATE I



FIG. 1. *Refractive index photograph taken after eight minutes at 13,410 r.p.m. in the ultracentrifuge showing sedimenting boundary of influenza virus A in 0.85% sodium chloride solution at pH 7.0. Direction of sedimentation from right to left. Concentration of virus solution 2 mgm. per ml.*



system. A description of the centrifuge has been given by Rhodes and van Rooyen (13).

In eight different preparations of purified virus one ID<sub>50</sub> for embryonated eggs contained  $10^{-14.3}$  to  $10^{-15.4}$  gm. of nitrogen.\* For five of these preparations the results fell between the limits  $10^{-14.85}$  to  $10^{-15.10}$  gm. nitrogen per ID<sub>50</sub>. The influenza virus particle was computed to have a weight of approximately  $10^{-15.2}$  gm. assuming a spherical shape of diameter 100 m $\mu$  and a density of 1.22 gm. per cc. Since Taylor (14) has found the virus to contain 10.0% nitrogen it was calculated from the above infectivity figures that the purified preparations contained, on the average, about 16 virus particles in one ID<sub>50</sub>. Friedewald and Pickels (3) determined about 10 particles per ID<sub>50</sub> in their purified preparations of PR8 virus.

#### *Incorporation of Radioactive Phosphorus into Influenza Virus*

In the experiments on the incorporation of radioactive phosphorus into the virus, P<sup>32</sup>, as inorganic phosphate, was diluted to the desired activity in sterile 0.85% saline adjusted to pH 7 with sodium hydroxide. Unless stated otherwise, 0.2 ml. of the solution of P<sup>32</sup> was injected into the allantoic cavity of the embryonated egg at an arbitrarily chosen interval three hours following inoculation of influenza virus by the same route. After 48 hr. incubation at 36° C., the allantoic fluid was harvested and the purification of the virus followed the general procedure already given.

Having ascertained that under these conditions the purified virus suspensions contained measurable amounts of P<sup>32</sup>, it was necessary to ensure that the P<sup>32</sup> was, in fact, closely associated with the virus and could not be removed by repeated washing of the elementary bodies. The following experiment was designed to settle this point. Total phosphorus and P<sup>32</sup> estimations were carried out on a number of the fractions obtained during the preparation of the purified radioactive virus and are reported in Table I. The specific activity is defined as the ratio of P<sup>32</sup> in c.p.m. to total phosphorus in  $\mu\text{gm}$ .

Two hundred and nine 11-day embryonated eggs were inoculated with influenza virus. Three hours later each egg received 91,200 c.p.m. of P<sup>32</sup>, contained in 0.2 ml. of 0.85% saline. During the ensuing 48 hr. incubation period 61 embryos died and were discarded. The remaining eggs were harvested and the allantoic fluid was allowed to stand overnight at 5° C. The agglutinated cells were removed by centrifugation and the supernatant solution (Supernatant A) was discarded. After washing the cells with Ringer - calcium chloride solution, the wash liquid (Supernatant B) was discarded. Fresh Ringer - calcium chloride solution was added and the virus eluted from the cells which were then removed by centrifugation and discarded. This virus solution was centrifuged at 20,000 g for one hour (Supernatant C), and the pellet resuspended in Ringer - calcium chloride and centrifuged at

\* These figures were given in error in the preliminary paper as  $4 \times 10^{-15}$  to  $5 \times 10^{-14}$  gm. nitrogen; they should have read  $4 \times 10^{-16}$  to  $5 \times 10^{-15}$  gm. nitrogen.

TABLE I

## SPECIFIC ACTIVITY OF RADIOACTIVE VIRUS DURING PURIFICATION PROCEDURE

Fraction	Volume of fraction, ml.	Total P <sup>32</sup> c.p.m.	Specific activity of virus, c.p.m./μgm. P
Supernatant A	1010	6,868,000	—
Supernatant B	101	47,000	—
Supernatant C	95	26,000	—
Virus suspension 1	9.5	3850	15.2
Supernatant D	7.6	225	—
Virus suspension 2	7.6	2340	15.0
Supernatant E	5.5	32	—
Virus suspension 3	5.5	1465	16.6
Supernatant F	3.5	Trace	—
Supernatant G	3.5	Trace	—
Supernatant H	3.5	Trace	—
Virus suspension 6	3.5	612	15.9

2000 g for 10 min. to remove large particles and agglutinated virus. The resulting supernatant solution (Virus suspension 1) was subjected to two similar cycles of high and low speed centrifugation to give Supernatants D, E, and Virus suspensions 2, 3, the third virus suspension being made in 0.85% saline. Supernatant F was obtained by centrifuging this suspension at 20,000 g, the virus pellet was resuspended in saline containing 0.01 M phosphate buffer, pH 7.0, and allowed to stand 48 hr. at 5° C. to permit any exchange of the P<sup>32</sup> of the virus with the buffer phosphate. Following centrifugation at 20,000 (Supernatant G) the virus was resuspended in saline, sedimented at high speed (Supernatant H), resuspended in saline, and finally centrifuged for 10 min. at 2000 g to remove the larger particles. The supernatant solution was Virus suspension 6 shown in Table I.

It can be seen from Table I that despite the repeated washings received by the elementary bodies and the opportunity allowed for exchange of P<sup>32</sup> to occur with phosphate buffer, the specific activities of the various virus suspensions remained essentially constant. This finding indicated that the P<sup>32</sup> was firmly fixed in the virus. Similar results were obtained in three further such experiments.

#### *Control Experiments on Addition of Radioactive Phosphorus to Infectious Allantoic Fluid*

The previous experiment demonstrated that influenza virus grown in the embryonated egg in the presence of P<sup>32</sup> contained a definite amount of the isotope. There was the possibility, however, that the isotope had not been incorporated into the virus during its actual growth in the cell, but after the virus had been liberated from the cells of the membrane into the allantoic fluid. As described later, a considerable amount of the injected P<sup>32</sup> remained

in the allantoic fluid, even at the end of the 48 hr. incubation period. Since maximum growth of the virus is almost complete in the first 24 hr. after infection (6), a considerable proportion of the freshly liberated virus would remain in contact with the radioactive allantoic fluid under conditions favorable to exchange of the isotope with the virus phosphorus. To gain information on this point an experiment was carried out in which  $P^{32}$  was added to freshly harvested infectious allantoic fluid from which the virus was subsequently isolated and its isotope content determined.

Allantoic fluid was collected from 97 embryonated eggs which had been infected with influenza virus two days previously and allowed to incubate in the usual way. Care was taken to exclude red blood cells when harvesting the fluid. Radioactive phosphate was added to the fluid to give 75,000 c.p.m. per ml., an amount corresponding to that used in the previous experiments on labelling the virus. After standing two days at 5° C. to allow any exchange to take place, chicken red cells were added to make a final 2% suspension. The virus suspension, obtained in the usual way by elution from the cells into saline, was subjected to four cycles of differential centrifugation, the resuspended virus after each step being analyzed for total P and  $P^{32}$  as shown in Table II.

TABLE II

SPECIFIC ACTIVITY OF VIRUS AFTER ADDITION OF  $P^{32}$  TO INFECTIOUS ALLANTOIC FLUID

Fraction	Volume of fraction, ml.	Total $P^{32}$ , c.p.m.	Specific activity of virus, c.p.m./ $\mu$ gm. P
Infectious allantoic fluid	471	35,200,000	—
Supernatant from agglutinated red cells	460	32,800,000	—
Wash liquid from agglutinated red cells	46	742,000	—
Supernatant 1 after 20,000 g	45.5	120,900	—
Virus suspension 1	13.6	374	2.9
Supernatant 2 after 20,000 g	11.6	276	—
Virus suspension 2	11.6	24	0.3
Supernatant 3 after 20,000 g	9.6	0	—
Virus suspension 3	9.6	48	0.7
Supernatant 4 after 20,000 g	7.6	0	—
Virus suspension 4	7.6	0	—

It is seen that the virus suspension contained a negligible amount of radioactivity as evidenced by its specific activity of 0.3 c.p.m./ $\mu$ gm. P. Such small amounts of radioactivity as were contained in this suspension, where the counting rate was two to three counts above background, were difficult to estimate with any accuracy. It is apparent, however, that little or no exchange had taken place between the virus and inorganic radioactive phosphate. Three further such experiments yielded similar results.

*Control Experiments on Addition of Radioactive Phosphorus to Purified Influenza Virus*

Although the previous control experiments indicated that influenza virus in infectious allantoic fluid did not take up P<sup>32</sup> *in vitro* from radioactive phosphate, it was thought of interest to add relatively large amounts of P<sup>32</sup> to a purified virus suspension to determine the efficiency of the differential centrifugation procedure in removing the isotope.

A suspension of influenza virus in saline was prepared in the usual way with two cycles of differential centrifugation. Radioactive phosphate was added to the purified virus to give a final concentration of 69,600 c.p.m. per ml. of suspension. After standing 24 hr. at 5° C. to permit any exchange, or adsorption of P<sup>32</sup> on the virus particles, the virus was sedimented at 20,000 g. The supernatant was decanted (Supernatant 1), a small quantity of saline was added to wash down the tube and quickly poured off, and the pellet resuspended in saline. This procedure was repeated three times; total phosphorus and P<sup>32</sup> estimations were carried out on the various fractions, the results being shown in Table III.

TABLE III

SPECIFIC ACTIVITY OF VIRUS AFTER ADDITION OF P<sup>32</sup> TO PURIFIED SUSPENSION

Fraction	Volume of fraction, ml.	Total P <sup>32</sup> , c.p.m.	Specific activity of virus, c.p.m./ $\mu$ gm. P
Virus suspension with added P <sup>32</sup>	4.6	320,100	—
Supernatant 1 after 20,000 g	4.6	288,500	—
Virus suspension 1	13.8	3160	23.5
Supernatant 2 after 20,000 g	13.8	2900	—
Virus suspension 2	11.8	73	0.74
Supernatant 3 after 20,000 g	11.8	Trace	—
Virus suspension 3	8.5	83	0.89
Virus suspension 4	6.5	51	0.79

From the specific activity figures it is apparent that little of the added P<sup>32</sup> remained in the second virus suspension. A small amount of radioactivity remained in the virus and was difficult to remove, but too much confidence cannot be placed in the radioactivity estimations for the second, third, and fourth virus suspensions because of the very low counting rate. A further such experiment gave similar results.

*Specific Activity of Influenza Virus Grown in Presence of Different Amounts of Radioactive Phosphorus*

Fig. 2 shows the variation in specific activity of influenza virus when grown in the embryonated egg in the presence of different amounts of P<sup>32</sup>. One experiment was carried out to illustrate this point. Two groups of 11-day embryonated eggs were inoculated with influenza virus then with P<sup>32</sup> after

three hours, 14,850 c.p.m. in each egg in the first group and 64,500 c.p.m. in the second. The remaining data were gathered incidentally from eight other experiments performed during the course of the work. In every case the

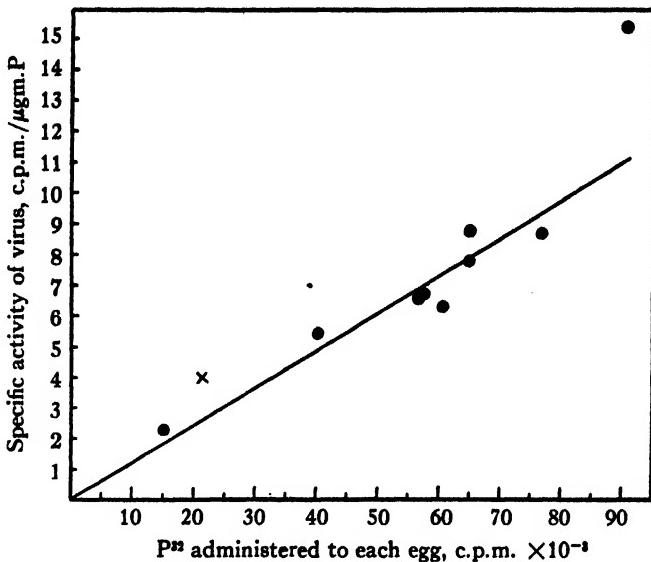


FIG. 2. Relationship of specific activity of purified radioactive virus to amount of radioactive phosphorus administered.

specific activity of the virus was determined after purification in the usual way with two cycles of differential centrifugation, except for one preparation which received only one cycle of centrifugation. The latter preparation is represented by a cross in the figure.

It is seen that the specific activity of the virus rises in a linear manner with the amount of injected P<sup>32</sup> up to at least 91,200 c.p.m. in each egg.

#### *Effect of β-Radiation from Radioactive Phosphorus on the Embryo and on the Growth and Properties of Influenza Virus*

During the course of the work it was found that the death rate of embryonated eggs injected with influenza virus A by the allantoic route might be as high as 6% during the succeeding 48 hr. incubation period and on the average was about 3-4%. When radioactive phosphorus was injected also, by the allantoic route, the embryo death rate increased until, with 70,000 c.p.m. injected into each egg, 10 to 30% of the embryos died; there was a large variation in the number of embryo deaths from one experiment to another. It seems probable that many of these deaths resulted from the effect of β-radiation, the dead embryos often appearing markedly haemorrhagic. This finding put a practical upper limit on the amount of isotope which could be injected into each egg and, consequently, an upper limit on the amount of isotope which could be incorporated into the influenza virus.

In spite of the fact that, in several experiments, many of the embryos died from radiation injury, the yield of influenza virus from the surviving eggs was no less than from eggs which had not received P<sup>32</sup>. This was confirmed on several occasions by doing infectivity titrations and CCA tests on the pooled allantoic fluid from eggs which had survived the injection of up to 150,000 c.p.m. of P<sup>32</sup>.

In addition, the properties of purified influenza virus containing radioactive phosphorus, up to a specific activity of 15 c.p.m./ $\mu$ gm. P did not seem to be measurably different from those of normal virus. The appearance in electron micrographs and the sedimentation constant were unchanged. Infectivity figures given in an earlier section for eight different preparations of purified virus include three radioactive preparations; there was no significant difference between the radioactive and normal virus preparations.

It would appear, therefore, that relatively severe irradiation of the allantoic membrane, where influenza virus multiplication is presumed to occur, did not interfere appreciably with the virus growth, nor did it alter the properties of the purified virus.

#### *Specific Activity of Influenza Virus with Varying Intervals Between Virus and Radioactive Phosphorus Inoculation into Embryonated Eggs*

Previous experience had indicated that the maximum rate of growth of the PR8 strain of influenza virus A occurred during 24 hr. after inoculation of the egg. It was assumed that if P<sup>32</sup> were injected into the allantoic cavity while the virus was rapidly growing, conditions should be favorable for incorporation of the isotope into the virus, since the cells of the allantoic membrane should be in intimate contact with P<sup>32</sup> during this period. Consequently, in all experiments involving labelling of influenza virus with P<sup>32</sup> so far reported in this paper, radioactive phosphorus was injected into embryonated eggs three hours following inoculation of virus.

There was a possibility, however, that P<sup>32</sup> uptake by the growing virus might be much greater if some of the normal phosphorus containing constituents in the membrane cells were labelled through injection of the isotope at some time prior to infection with influenza virus. Therefore, a single experiment was carried out in which a group of 50 embryonated eggs was inoculated with P<sup>32</sup> at each of the intervals, 48 hr. before, 24 hr. before, a few minutes after, and 24 hr. after infection with 10-100 ID<sub>50</sub> of influenza virus. Each egg of the four groups received 76,000 c.p.m. of the isotope. After incubation of the eggs the virus from each group was purified, as usual, analyzed for P<sup>32</sup> and total phosphorus, and the specific activity was calculated. The results are shown in Table IV.

It would appear that when P<sup>32</sup> was injected into eggs 48 hr. previous to virus infection, the specific activity of the virus was about 22% higher than when the isotope was administered almost simultaneously with the virus. When the isotope was injected 24 hr. after infection the specific activity of the virus was very low, and it is worth noting that this finding supports

strongly the previous indication that little or no exchange occurs between virus and P<sup>32</sup>. In this case the virus, freshly liberated from the allantoic membrane during the 24 hr. following infection, remained in contact, *in vivo*,

TABLE IV

SPECIFIC ACTIVITY OF INFLUENZA VIRUS WHEN P<sup>32</sup> INJECTED  
INTO EMBRYONATED EGGS AT DIFFERENT TIMES WITH  
RESPECT TO TIME OF VIRUS INOCULATION

Time of injection of P <sup>32</sup>	Specific activity of purified virus c.p.m./ $\mu$ gm. P
48 hr. before virus	10.5
24 hr. before virus	9.8
Same time as virus	8.6
24 hr. after virus	0.56

with allantoic fluid containing large amounts of P<sup>32</sup> for a further 24 hr. The low specific activity of the virus from this group of eggs, compared to the very much higher specific activities obtained in the other three groups, indicates that the virus is labelled with the isotope only during its growth in the cells of the membranes. Even the low specific activity observed in the fourth group could be accounted for by the relatively small amount of virus multiplication which occurred during the second 24 hr. period following infection of the eggs.

*Disappearance of Radioactive Phosphorus from Allantoic Fluid and from Yolk Sac of Embryonated Egg*

During the course of the work it was felt necessary to obtain information on the amount of P<sup>32</sup> remaining in the allantoic fluid at various intervals following injection of P<sup>32</sup>. Experiments designed for this purpose were carried out as follows:

Each of a number of 11-day embryonated eggs was injected with 0.2 ml. of P<sup>32</sup> solution by the allantoic route, the isotope being dissolved either in saline or in 0.1 M phosphate buffer, pH 7. Within 5 to 10 min. after injection the allantoic fluid of five eggs was harvested and the total volume of fluid measured. Radioactivity estimations were carried out on the combined fluids. Control experiments, carried out by diluting a saline solution of known P<sup>32</sup> content in normal allantoic fluid and measuring the activities of various dilutions, indicated that the presence of allantoic fluid did not interfere with the method of assay. The remaining eggs were incubated at 36° C. and, at intervals, groups of five eggs each were treated in the same manner as above.

In all, 10 such experiments were performed, the amount of P<sup>32</sup> injected per egg varying between 8700 c.p.m. to 40,900 c.p.m. from one experiment to

another. The results of two of the experiments, which are representative of the remainder, are shown in Fig. 3.

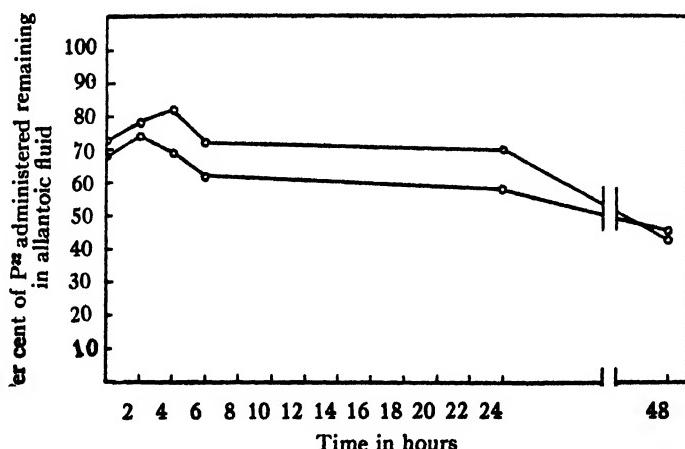


FIG. 3. *Rate of disappearance of P<sup>32</sup> from the allantoic fluid of 11-day embryonated eggs.*

In none of the experiments was more than 90% of the administered P<sup>32</sup> recovered from the groups of eggs harvested at zero time. This was probably partly due to the fact that it was impossible to harvest the allantoic fluid quantitatively. The slight increase in P<sup>32</sup> recovered in the allantoic fluid, shown between two to four hours in Fig. 3, was observed in each of the remaining eight experiments, although in some cases the increase was not seen until four to six hours after injection of isotope. These findings were not considered of immediate importance and were not investigated further. In two experiments, 42% and 51% of the administered isotope was recovered after 72 hr.

Although similar experiments were not done with influenza virus infected eggs, it was observed incidentally during another part of the work that 40% to 50% of the administered P<sup>32</sup> was recovered after 48 hr. from the allantoic fluid of infected eggs.

Two experiments were carried out to determine whether P<sup>32</sup> injected into the yolk sac of 11-day embryonated eggs would appear rapidly in the allantoic fluid. Since the technique of inoculation into the yolk sac is not easy it was necessary to ensure that the isotope was placed in the desired position. Consequently the P<sup>32</sup> solution, 46,000 c.p.m. into each egg, was made up in 2% trypan red solution for inoculation. It had been determined previously that trypan red remained indefinitely in the yolk sac and that it was innocuous to the developing embryo. On harvesting the eggs the allantoic fluid was used only from those eggs in which the dye was localized in the yolk sac.

After 72 hr., only 1.5% of the administered P<sup>32</sup> had appeared in the allantoic fluid.

### Discussion

It is considered that the suspensions of purified virus handled throughout this work consisted almost entirely of the elementary bodies of influenza virus. Sedimentation (3, 15), electron microscope (15, 17), infectivity results (3), and chemical analysis (5, 14) were in accord with the observations of previous workers and together suggested that little or no soluble or particulate matter other than virus was present in these preparations.

It would appear that the P<sup>32</sup> in the radioactive virus was, in fact, incorporated into the structure of the virus. This is indicated by the findings that virus does not exchange with P<sup>32</sup> and that the isotope in labelled virus cannot be removed by repeated washings of the elementary bodies. Further evidence was offered by the observation, made during chemical analysis of radioactive virus, that both the phospholipid and nucleic acid fractions of purified virus contained P<sup>32</sup> (5).

In radioactive tracer experiments with biological materials, it is essential that the radiation from the isotope should not interfere with the metabolism of the system under observation. In such studies, it is generally easy to exclude radiation effects since only minute amounts of tracer are required. However, for the purpose of further study of the labelled influenza virus, it was necessary to obtain virus with high specific radioactivity. It became apparent early in the work that in order to achieve this end, relatively large amounts of P<sup>32</sup> would have to be injected into the virus infected egg. In many experiments, when 450-600  $\mu$ rd. of P<sup>32</sup> or more were injected into each egg, a significant proportion of the embryos died.

It was considered that these deaths may have resulted from some toxic material present in the P<sup>32</sup>. However, the isotope solutions, as received from the National Research Council, were usually diluted at least one thousandfold before administration to the eggs in 0.2 ml. amounts. It was thought improbable that any compound would be present in sufficiently high concentration in the original solution to cause the high mortality observed in some of the groups of fertile eggs. It was therefore presumed that death of the embryos was in large measure due to  $\beta$ -radiation injury.

In spite of these observations it is noteworthy that in the infected eggs which survived the injection of as much as 1300  $\mu$ rd. of P<sup>32</sup> the yield of virus was not appreciably less than from eggs which had not received the isotope. It was noted also that the specific activity of purified radioactive virus rose linearly with increasing amounts of P<sup>32</sup> administered to the infected eggs up to at least 775  $\mu$ rd. for each egg. If radiation were interfering with the growth of virus both the yield and specific activity of the virus might be expected to decrease rapidly with increasing amounts of isotope administered. It would thus appear that the mechanisms involved in the growth of influenza virus are relatively resistant to  $\beta$ -radiation.

One of the main points we had hoped to investigate with the radioactive virus was the fate of the isotope when the virus was growing in the allantoic

membrane. So far such a study has been precluded by the small amount of  $P^{32}$  which has been introduced into the virus. It can be calculated that the specific activity of the radioactive virus would have to be increased at least a thousandfold over the highest value yet obtained in order to study this problem to any advantage. While there would seem to be little hope of obtaining influenza virus with such a high specific activity by the methods reported here, it is a subject for investigation whether the activity might be increased substantially by administering the isotope by some route other than by the allantoic sac.

The infectivity, sedimentation characteristics, and morphology of the purified radioactive virus were not significantly different from those of unlabelled virus. It was calculated that, for influenza virus having a specific activity of 1.29  $\mu$ rd. per  $\mu$ gm. P (15 c.p.m. per  $\mu$ gm. P), the proportion of radioactive phosphorus atoms to total phosphorus atoms in the virus was 1 to  $8.4 \times 10^9$ . That is, on the average, one radioactive phosphorus atom was present for every 66,500 virus particles. Therefore, even if the properties of labelled virus were markedly different from those of unlabelled virus, the presence of such a small proportion of radioactive virus particles would hardly be expected to cause a measurable change in the biological or physical properties of the virus preparation. Thus it is not possible to state as yet whether labelled virus behaves in the same manner as unmarked virus.

### Acknowledgments

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## THE EFFICACY OF VARIOUS THIOLS AS ANTIDOTES TO LEWISITE<sup>1</sup>

By S. D. SIMPSON<sup>2</sup> AND L. YOUNG<sup>3</sup>

### Abstract

The following thiols were tested for their effectiveness in counteracting the systemic toxic action of lewisite ( $\beta$ -chlorovinylidichloroarsine) in the rat: 1- and 2-mercaptopropane, 2-mercaptopropanol, 1,2-dimercaptoethane, 1,2- and 1,3-dimercaptopropane, 1,2,3-trimercaptopropane, 1,2-dimercapto-*n*-butane, 2,3-dimercaptopropanol, 1,3-dimercapto-2-propanol, 2,2'-dimercaptodiethyl ether, 2,2'-dimercaptodiisopropyl ether, and 3,3'-dimercaptodipropyl ether. The toxicity and volatility of these compounds were also studied.

The tests of antidotal activity were conducted by applying the thiol to the skin at a site which had been dosed with approximately twice the LD<sub>50</sub> of lewisite. Under these conditions the monothiols gave no evidence of antidotal activity whereas all the dithiols and the trithiol studied showed some activity. 2,3-Dimercaptopropanol (BAL) and its isomer, 1,3-dimercapto-2-propanol, were much more effective as antidotes to lewisite than any of the other compounds tested. Not only did 2,3-dimercaptopropanol show a somewhat higher antidotal activity than its isomer, but it was also found to be much less toxic.

### Introduction

The view that the toxic action of trivalent arsenical compounds is related to their reaction with essential thiol compounds in the organism was advanced by Voegtlin, Dyer, and Leonard (11) in 1923. These workers also found that under suitable conditions certain monothiols can diminish or prevent the toxic action of arsenical compounds (12) and this finding was supported by results obtained in other investigations (1, 4). The outbreak of World War II provided a stimulus to the search for antidotes to arsenical compounds, and in particular to chemical warfare agents such as lewisite ( $\beta$ -chlorovinylidichloroarsine). In 1940, Peters, Stocken, and Thompson, in the course of a study of the interaction of lewisite and the thiol groups of keratine, found that most of the arsenic in the derived protein was combined with two thiol groups and they were led to the conclusion that "simple dithiol compounds might form relatively stable ring compounds with lewisite and other trivalent arsenical compounds and so compete successfully with 'dithiol' proteins in the tissues" (3). This, in turn, led them to the important finding that the dithiol, 2,3-dimercaptopropanol (later named BAL, British Anti-Lewisite, in the United States) is highly effective in preventing the local and systemic

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actions of lewisite. Reviews of work on BAL have been published by Peters, Stocken, and Thompson (3), Peters (2), Thompson (10), and Stocken and Thompson (8). Work on BAL in the United States has been described by Waters and Stock (13) and Sulzberger and Bair (9), and Canadian researches on BAL have been reviewed by Young (15).

The work described herein was carried out in 1942-43 (7) following the receipt of early accounts of the researches of the Oxford workers. It was undertaken in order to compare the toxicity of BAL and its antidotal activity in lewisite poisoning with those of a series of related compounds. For this purpose the following thiols were synthesized by Simpson (5, 6): 1,2-dimercaptoethane, 1,2-dimercaptopropane, 1,3-dimercaptopropane, 1,2,3-trimercaptopropane, 1,2-dimercapto-*n*-butane, 1,3-dimercapto-2-propanol, 2,2'-dimercaptodiethyl ether, 2,2'-dimercaptodiisopropyl ether, and 3,3'-dimercapto-dipropyl ether. These compounds, together with 1- and 2-mercaptopropane, 2-mercptoethanol, and 2,3-dimercaptopropanol, were tested for their toxicity and their activity as antidotes to lewisite. As the main method of administration of an antilewisite compound in the field appeared likely to be by direct application to the site of contamination, the tests of toxicity and antidotal activity were conducted by applying the thiols to the skin. Among the factors which influenced the results obtained under these conditions was the volatility of the compounds being tested, and for this reason all the animal tests were performed in a room maintained at  $25 \pm 1^\circ\text{C}$ . In order that the volatility of the thiols might be taken into consideration in the interpretation of the results of the tests of antidotal activity, a quantitative study was made of the evaporation of the thiols at  $25^\circ$  and  $37^\circ\text{C}$ .

### Materials and Methods

The 1- and 2-mercaptopropane and the 2-mercptoethanol used were products of the Eastman Kodak Company, the 2,3-dimercaptopropanol was provided by the Directorate of Chemical Warfare, Department of National Defence, Ottawa, and the remaining nine thiols were synthesized by Simpson (5, 6). The sample of lewisite used had been carefully purified and was colorless (b.p.  $79.5\text{--}80.5^\circ$  at 14 mm. pressure;  $n_D^{20} = 1.6091$ ;  $d_4^{25} = 1.883$ ). It was kindly provided by Dr. G. F. Wright, Department of Chemistry, University of Toronto.

White rats, all of the same strain, from the colony of the Department of Biochemistry, University of Toronto, were used in the animal experiments. Equal numbers of male and female rats were used under each set of conditions studied. Each rat weighed between 150 and 185 gm. In every test the site of application of the agent or agents was the skin of the mid-lumbar region. Before the animal was dosed it was anesthetized with ether and the hair in the lumbar region was cut as closely to the skin as possible by means of electric shears and scissors. The temperature of the room in which the dosing experiments were performed was maintained at  $25 \pm 1^\circ\text{C}$ . At the end of the dosing operation each rat was placed in a separate metal cage in which it was

provided with food (Master Fox Breeding Ration—Toronto Elevators Limited) and water *ad libitum*. The animal was then weighed daily for a week and if it was gaining weight at the end of this period it was classed as having survived. If it was losing weight at this time it was kept under observation for a further period to determine whether it would recover.

In order to determine the toxicity of a thiol the compound was measured on to the skin of the anesthetized animals from a pipette with 0.01 ml. graduations, and was rubbed into the skin for five minutes with the flattened end of a glass rod. Anesthesia was continued for 30 min. after dosing. The toxicity of lewisite was determined under similar conditions, except that this agent was applied by means of a micropipette of the type described by Young (14, 16) and was not rubbed into the skin. In testing the antidotal activity of a thiol, each rat in the test group was anesthetized with ether and was dosed on the skin with 40 mgm. of lewisite per kgm. body weight (approximately  $2 \times LD_{50}$ —see below). After a measured interval of time, 0.05 ml. of the thiol was applied to the contaminated area and was rubbed into the skin for five minutes. In those experiments in which the interval between the application of the lewisite and the thiol was 15 or 30 min. the animals were kept anesthetized until after the thiol had been applied. When the interval was one hour or longer, the animals were allowed to regain consciousness 30 to 45 min. after they had been dosed with lewisite and were anesthetized again before they were treated with the thiol.

The procedure used for measuring the evaporation of a thiol was as follows. By means of a pipette, 0.16 ml. of the thiol was measured on to a microscope coverslip supported on a watch glass and its weight was determined immediately. The amounts of thiol which evaporated in 15, 30, and 60 min. were then determined by weighing. The evaporation at 25° C. was measured in a room maintained at that temperature. All the weighings were made on an oil-damped balance and each weighing was completed in less than one minute. The measurements at 37° C. were made by placing the microscope coverslip carrying the thiol in a large incubator for 15, 30, or 60 min., and then weighing it on the oil-damped balance within one to two minutes after removal from the incubator. All determinations were made in duplicate.

## Results

### Toxicity of the Thiols

The results obtained in studies of the toxicity of the thiols are shown in Table I. In many experiments the administration of the thiol gave rise to profuse lachrymation. Most of the rats which received lethal doses of thiol died within 24 hr. after dosing, and death was often preceded by violent convulsions. Some of the animals died several days after being dosed, and during this period they lost weight and at the time of death they were very emaciated. The rats which received sublethal doses of thiol usually lost weight for a few days and then rapidly gained in weight. Those rats which were

TABLE I

## THE TOXICITY OF THIOLS WHEN APPLIED TO THE SKIN OF THE RAT

Thiol	mM. of thiol per ml.	Dose of thiol applied (ml./kgm. body wt.)						
		0.3	0.6	0.9	1.2	1.8	2.4	3.0
Rats surviving in each group of four rats								
1-Mercaptopropane	11.0	-	-	-	-	-	-	4
2-Mercaptopropane	10.6	-	-	-	-	-	-	4
2-Mercaptoethanol	14.4	-	4	3	2	3	0	1
1,2-Dimercaptoethane	11.9	-	-	4	3	0	-	-
1,2-Dimercaptopropane	9.8	-	4	3	3	3	1	1
1,3-Dimercaptopropane	10.0	-	-	-	4	3	2	0
1,2,3-Trimercaptopropane	8.8	3	1	0	-	-	-	-
1,2-Dimercapto-n-butane	8.5	-	4	3	2	0	-	-
2,3-Dimercaptopropanol	10.0	-	4	4	4	3	0	0
1,3-Dimercapto-2-propanol	10.0	1	0	-	-	-	-	-
2,2'-Dimercaptodiethyl ether	8.1	-	4	2	1	0	-	-
2,2'-Dimercaptodiisopropyl ether	6.3	-	-	4	2	0	-	-
3,3'-Dimercaptodipropyl ether	6.3	-	-	4	2	0	-	-

dosed with amounts of thiol approaching the lethal level showed the greatest loss of weight. For several days after being dosed some animals were hyperexcitable when handled.

*Toxicity of Lewisite*

The data obtained in the determination of the toxicity of lewisite when applied to the skin of the rat are shown in Table II. The animals which

TABLE II

## THE TOXICITY OF LEWISITE WHEN APPLIED TO THE SKIN OF THE RAT

No. of rats in group	Av. wt. of rats in group, gm.	Mgm. of lewisite applied	Dose of lewisite mgm./kgm. body wt.	Per cent mortality
10	170	1.0	6	0
10	170	2.0	12	0
10	168	3.0	18	30
10	173	4.0	23	90
10	169	5.0	30	100

received lethal doses of lewisite usually developed pleural effusion and severe diarrhoea, and they rarely survived for longer than 24 hr. after being dosed. From the data in Table II it appears that the LD<sub>50</sub> of the sample of lewisite used in the present work was between 18 and 23 mgm. per kgm. body weight.

*Antidotal Activity of the Thiols*

The results obtained in studies of the efficacy of the thiols as antidotes to lewisite are shown in Table III.

TABLE III

THE ANTIDOTAL ACTIVITY OF THIOLS APPLIED CUTANEOUSLY TO RATS DOSED WITH  
40 MG.M. LEWISITE PER KG.M. (APPROX. 2 X LD<sub>50</sub>)

Thiol	mM. of thiol applied (0.05 ml.)	Interval in minutes between application of lewisite and application of thiol						
		15	30	60	90	120	180	240
		Rats surviving in each group of four rats						
1-Mercaptopropane	0.55	0	-	-	-	-	-	-
2-Mercaptopropane	0.53	0	-	-	-	-	-	-
2-Mercaptoethanol	0.72	0	-	-	-	-	-	-
1,2-Dimercaptoethane	0.60	4	2	0	-	-	-	-
1,2-Dimercaptopropane	0.49	3	1	0	-	-	-	-
1,3-Dimercaptopropane	0.50	4	3	1	-	-	-	-
1,2,3-Trimercaptopropane	0.44	4	4	1	-	-	-	-
1,2-Dimercapto-n-butane	0.43	2	1	0	-	-	-	-
2,3-Dimercaptopropanol	0.50	4	4	4	4	3	3	1
1,3-Dimercapto-2-propanol	0.50	3	4	4	4	4	0	-
2,2'-Dimercaptodiethyl ether	0.41	4	4	2	-	-	-	-
2,2'-Dimercaptodiisopropyl ether	0.32	4	4	0	-	-	-	-
3,3'-Dimercaptodipropyl ether	0.32	4	4	1	-	-	-	-

Each animal was dosed with 40 mgm. of lewisite per kgm. body weight. This was approximately twice the LD<sub>50</sub>, and every rat in a control group of 16 animals died within 24 hr. of being dosed with this amount of the agent. The amount of thiol used in each test of antidotal activity was 0.05 ml., i.e. approximately 0.30 ml. per kgm. weight. This was a sublethal dose for every thiol tested except 1,2,3-trimercaptopropane and the highly toxic dithiol, 1,3-dimercapto-2-propanol. It appeared, however, that the use in all experiments of a volume of thiol which corresponded to a sublethal dose of 1,3-dimercapto-2-propanol would have necessitated reducing the amount to a level which would have been unsatisfactory for testing the antidotal activity of many of the thiols. It is interesting to note that there were 19 survivors out of 20 rats dosed with lewisite and treated at intervals up to two hours with 1,3-dimercapto-2-propanol (see Table III), a finding which suggests that the level of this thiol which can be tolerated is increased in animals dosed with lewisite. Those rats which died after being dosed with lewisite and treated with a thiol usually survived no longer than 24 hr. after being dosed. The animals which recovered usually lost weight for several days after being dosed and thereafter they gained in weight rather slowly.

The most effective antidotes were those thiols which evaporated slowly, and the following control experiment was carried out in order to find whether protection against lewisite was afforded by the mere application of a liquid with low volatility to the site of contamination. The agent selected was glycerol, because of its physical characteristics, its lack of toxicity, and its chemical relationship to the most effective antidotes tested, namely the dimercaptopropanols. Eight rats were dosed with 40 mgm. of lewisite per

kgm. body weight and 15 min. later 0.05 ml. of glycerol was applied to the site of contamination on four of the animals and was rubbed into the skin for five minutes. The remaining four rats were treated with glycerol in a similar manner 30 min. after being dosed with lewisite. All eight rats died within 24 hr. after being dosed.

### *Evaporation of the Thiols*

The purpose of the experiments in which the evaporation of the thiols was studied was merely to obtain evidence of any gross differences in the volatilities of the thiols.

The evaporation of the thiols was measured at 25° and 37° C. because these were the extreme temperatures, i.e. room temperature and body temperature of the rat, to which the thiols could possibly have been subjected during the animal experiments. From the results obtained (see Table IV) it appears

TABLE IV  
THE EVAPORATION OF THIOLS IN STILL AIR AT 25° AND 37° C.

Thiol	25° C. Per cent (by wt.) of thiol which evaporated in:			37° C. Per cent (by wt.) of thiol which evaporated in:		
	15 min.	30 min.	60 min.	15 min.	30 min.	60 min.
1-Mercaptopropane	(100)	—	—	(100)	—	—
2-Mercaptopropane	(100)	—	—	(100)	—	—
2-Mercaptoethanol	8	17	36	17	32	59
1,2-Dimercaptoethane	28	61	(100)	58	(100)	—
1,2-Dimercaptopropane	49	91	(100)	64	(100)	—
1,3-Dimercaptopropane	11	23	46	24	45	88
1,2,3-Trimercaptopropane	1	2	4	2	4	7
1,2-Dimercapto- <i>n</i> -butane	28	59	96	48	87	(100)
2,3-Dimercaptopropanol	<1	<1	<1	1	2	3
1,3-Dimercapto-2-propanol	<1	<1	1	<1	1	2
2,2'-Dimercaptodiethyl ether	1	2	4	3	6	11
2,2'-Dimercaptodiisopropyl ether	2	4	8	3	6	12
3,3'-Dimercaptodipropyl ether	<1	<1	1	<1	1	2

that there were great differences in the rates at which the compounds evaporated. The possible significance of the volatility of certain of the thiols in relation to their effectiveness when applied cutaneously as antidotes to lewisite is discussed below.

### Discussion

The main conclusion which can be drawn from the present work is that 2,3-dimercaptopropanol (BAL), under the test conditions used, was more effective in preventing the systemic toxic action of lewisite than any of the other thiols studied. These compounds consisted of three monothiols, eight dithiols, and one trithiol.

The only thiol found to have an antidotal activity approaching that of 2,3-dimercaptopropanol was its isomer, 1,3-dimercapto-2-propanol. Both of these compounds proved to be effective antidotes even when their administration was delayed for two hours after contaminating rats with amounts of lewisite which would otherwise have caused death. These two compounds, however, showed a marked difference in toxicity. Not only was the 1,3-compound found to be much more toxic than its isomer, but it was also more toxic than any other thiol tested.

The monothiols, 1- and 2-mercaptopropane, were found to have no value as antidotes to lewisite under the test conditions used. These thiols were the most volatile of the compounds studied, and their lack of antidotal activity may possibly have been related to their rapid evaporation from the skin. It is noteworthy, however, that 2-mercptoethanol, the other monothiol studied, showed low volatility but gave no evidence of antidotal activity.

All the dithiols and the trithiol studied in the present work showed some degree of antidotal activity. Much higher activity was observed with 2,3-dimercaptopropanol and 1,3-dimercapto-2-propanol than with any of the other 1,2- and 1,3-dithiols studied, namely 1,2-dimercaptoethane, 1,2- and 1,3-dimercaptopropane and 1,2-dimercapto-*n*-butane. These latter compounds were found to be much more volatile than the dimercaptopropanols, and this may well have been one of the factors which gave rise to the observed differences in activity. An indication that the increase in antidotal activity brought about by the introduction of a hydroxyl group into 1,2- or 1,3-dimercaptopropane is not wholly related to a decrease in volatility is provided by a comparison of the activity and volatility of the dimercaptopropanols with those of 1,2,3-trimercaptopropane. The volatility of this latter compound was found to be close to that of the dimercaptopropanols, but as an antidote to lewisite it proved to be little better than 1,3-dimercaptopropane.

If the efficacy of a dithiol as an antidote to an arsenic compound is related to its ability to form a cyclic dithioarsinite, then the stability of the ring compound becomes a factor in determining the antidotal activity of the thiol (3). The stability of the ring compound is itself influenced by the nature and number of the constituents of the cyclic structure. Some interest attaches, therefore, to the antidotal behavior of the 1,5-dithiols, 2,2'-dimercaptodiethyl ether and 2,2'-dimercaptodiisopropyl ether, and the 1,7-dithiol, 3,3'-dimercaptodipropyl ether, for if these compounds react with trivalent arsenicals to form cyclic derivatives, the 1,5-dithiols give rise to eight-membered ring compounds, and the 1,7-dithiol forms a 10-membered ring compound. The dithiol ethers showed low volatility and when applied to the skin they appeared to be somewhat more effective as antidotes than those 1,2- and 1,3-dithiols which were rather volatile. On the other hand, they were much less active than the dimercaptopropanols. If there was formation of 8- and 10-membered ring compounds of the type described above, these may have been much less stable than the five- and six-membered rings formed by the interaction of lewisite and dimercaptopropanols. It must be borne

in mind, however, that even though formation of a stable cyclic dithioarsinite may favor the antidotal activity of a dithiol, such factors as the distribution, metabolism, excretion, and toxicity of the thiol and those of any derivatives which it forms with the arsenic compound may also greatly influence the outcome of its use as an antidote in arsenic poisoning.

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## THYROID FUNCTION IN ESSENTIAL HYPERTENSION<sup>1</sup>

By A. E. THOMPSON, NANCY E. MATHERS, AND W. F. PERRY

### Abstract

The functional condition of the thyroid glands of 32 hypertensive subjects was assessed by measurements of the plasma protein bound iodine. These did not differ significantly from those of a control group. No correlation was found within the hypertensive group between the severity of the disorder and the level of the plasma protein bound iodine. It was concluded that the activity of the thyroid gland is not affected by the presence of hypertension.

### Introduction

Investigations of the state of the thyroid gland in hypertension have chiefly depended on estimations of the basal metabolic rate. There seems to be general agreement that hypertensive subjects show a higher than normal incidence of elevated rates but as recently as 1947 Rosenkrantz and Marshall (7) considered that it was a matter of opinion whether this was due to hyperactivity of the thyroid gland or merely a reflection of increased cardiac work or complications such as congestive heart failure. Measurements of the total blood iodine have not clarified the situation as Turner *et al.* (10) found it low while Curtis and Fertman (1) found a tendency to elevation.

Plasma protein bound iodine values have been shown (3, 4) to bear a close relationship to the clinical status of patients with disordered thyroid function. It also appears that the values are not disturbed by extra thyroidal disorders except in the presence of hypoproteinemia (5), obesity (11), or medication with organic iodine compounds (3). Using this measurement Salter *et al.* (8) found normal values in four cases of hypertension but Perry and Cosgrove (4) reported elevated values in two of six cases. The present study was undertaken to reassess and extend these observations.

### Subjects and Methods

The subjects studied are listed in Table I, and were selected chiefly from the Outpatients' Department without regard to the degree of hypertension, its duration, or the complications. A special effort was made to include males while cases of clinically obvious thyroid dysfunction or obesity were excluded. The subjects include five of the six cases reported by Perry and Cosgrove. The sixth was a woman of 68 who on repeated examination had shown elevation of the plasma protein bound iodine. Following the appearance of their report, death occurred due to rupture of a myocardial infarct and at autopsy the thyroid gland was profoundly atrophic. Because of this and because of a history of prolonged medication with a variety of compounds which may have

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contained iodine it was decided that she could no longer be considered a simple case of hypertension. The other case stated to have had a high plasma protein bound iodine value has been reinvestigated and is reported here as case No. 5. The previous value of 14.0  $\mu\text{gm.}\%$  must be ascribed to a technical error.

The blood pressure was measured by the standard auscultatory procedure. Each resting pressure recorded in Table I was the mean of seven measurements made over a 45 min. period after 20 min. rest in the recumbent position, while the maximal pressure was the highest noted in the patient's chart. Gross enlargement of the heart was considered to be present when the apex beat was more than 10 cm. from the midsternal line or when a radiogram showed that its transverse diameter exceeded one-half the internal breadth of the chest.

Left ventricular strain was diagnosed by the standard electrocardiographic criteria. The severity of the changes in the ocular fundus were assessed according to the grading of Keith *et al.* (2). The basal metabolic rate was measured using the Benedict-Roth closed circuit equipment.

The plasma protein bound iodine was measured in duplicate on venous blood by the method of Perry and Cosgrove (4). In each case the total plasma protein measured by the method of Phillips *et al.* (6) was found to be within the normal range.

### Results

The mean protein bound plasma iodine of the 32 hypertensive subjects was  $4.9 \mu\text{gm.} \pm 1.5$  (S.D.) with a range of 1.9 to 9.0  $\mu\text{gm.}\%$ . In a previously reported (4) group of 34 normal subjects the mean was  $5.9 \mu\text{gm.}\% \pm 1.3$  (S.D.) with a range of 4.0 to 9.3  $\mu\text{gm.}\%$ . An analysis of variance (9) showed the difference between the groups to be of slight significance,  $P > .01$  and  $< .05$ .

The pertinent laboratory findings and clinical details of the hypertensive subjects are listed in Table I in ascending order of their plasma protein bound iodine concentrations. It will be seen that the subjects with the lowest values are similar to those with highest values in regard to resting blood pressure, maximal blood pressure, cardiac enlargement, electrocardiographic findings, ocular changes and symptoms. Thus it is clear that in the present series the protein bound iodine values cannot be correlated with any of the various criteria considered to reflect the severity or chronicity of the hypertensive condition.

### Discussion

The results reported here indicate that the plasma protein bound iodine values of the hypertensive subjects were similar or possibly slightly lower than those of healthy individuals. Such differences as were found may be due to the fact that the values for healthy individuals were obtained largely on males under the age of 35, whereas almost all the hypertensive subjects

TABLE I  
DETAILS OF HYPERTENSIVE SUBJECTS

No.	Sex	Age	Protein bound iodine, $\mu\text{gm}.\%$	Blood pressure				B.M.R.	Cardiac exam.*		Fundus**	Symptoms (duration in years)						
				Resting		Maximal			Size	Strain			Headache	Dyspnea	Angina	Failure***		
				Sys.	Dia.	Sys.	Dia.											
1	M	55	1.9	200	130	240	190	-10	0	+	2	0	0	0	0	0		
2	M	54	1.9	200	120	220	155		+	+	2	4	1	0	0	0		
3	M	85	3.4	190	110	210	125	0	+	+	3	0	5	0	0	0		
4	F	47	3.6	180	115	250	165	+10	+	+	1	0	7	0	0	6 (4)		
5	F	60	3.6	195	80	220	110	0	+	+	1	0	2	0	0	1 (1)		
6	M	45	3.6	180	120	240	140		+	0	2	2	0	0	0	0		
7	M	59	3.7	240	140	275	155		0	+	1	0	0	0	0	0		
8	F	65	3.8	200	115	210	125	+ 8	+	+	3	0	0	4	0	0		
9	F	45	4.1	220	130	250	140	0	+	0	1	0	4	0	0	0		
10	F	23	4.2	165	95	180	105	+ 8	0	0	0	0	0	0	0	0		
11	F	51	4.2	200	115	240	140	+ 1	+	+	3	1	6	0	0	0		
12	M	73	4.2	180	100	210	120		0	+	1	0	10	0	0	0		
13	F	49	4.4	240	145	260	160	- 5	0	0	9	8	0	0	0	0		
14	F	46	4.4	180	100	230	110	-12	0	0	2	2	2	0	0	0		
15	F	58	4.4	180	105	230	120	- 2	+	0	3	0	4	0	0	4 (2)		
16	F	68	4.4	185	100	205	140	- 3	+	+	2	8	0	3	0	0		
17	M	56	4.5	180	115	200	145	+18	0	+	2	6	0	0	0	0		
18	F	48	4.7	200	120	245	140	+10	0	+	1	8	0	0	0	0		
19	M	45	4.7	170	110	175	120	+ 6	0	0	0	0	0	0	0	0		
20	M	59	4.8	175	100	190	110		0	0	1	4	0	0	0	0		
21	M	31	4.9	200	130	250	170	+15	+	+	4	1	0	0	0	0		
22	F	56	5.0	170	100	240	120	- 8	+	+	2	0	6	5	0	0		
23	F	56	5.1	160	115	250	150	+10	+	+	0	1	0	0	1 (1)	0		
24	M	58	5.5	180	105	190	110		+	+	1	1	1	0	0	0		
25	F	57	5.7	130	85	240	150	+13	+	+	3	0	10	10	10 (1)			
26	F	64	5.8	180	80	250	110	0	+	+	1	0	10	10	1 (1)			
27	M	53	6.3	160	125	240	145	+ 5	+	+	1	0	1	0	0	1 (1)		
28	F	62	6.4	180	95	190	120	- 9	0	0	2	0	7	10	6 (1)			
29	F	22	6.9	160	115	260	190	- 4	0	0	1	0	0	0	0	0		
30	F	33	7.6	160	120	170	130	- 2	0	0	1	4	5	0	0	0		
31	F	47	7.7	220	120	280	155	+11	0	0	2	7	0	0	0	0		
32	M	48	9.0	190	100	260	165	+11	0	0	4	1	1	0	0	0		

\* Presence of gross cardiac enlargement and electrocardiographic evidence of left ventricular strain is indicated by a + sign.

\*\* Grade of hypertensive retinopathy.

\*\*\* Figure in brackets indicates number of episodes of congestive heart failure.

Note: Cases 1, 23, and 25 had a thyroidectomy five or more years previously. Cases 4, 5, 23, 24, 25, 26, and 27 were receiving digitalis.

were well beyond this age and females outnumbered the males. Furthermore, within the hypertensive group the severity of the condition bore no relation to the level of protein bound iodine. Hence it is concluded that the activity of the thyroid gland is not affected by the presence of essential hypertension.

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# THE EFFECT OF ADRENOCORTICOTROPHIC HORMONE AND SURGICAL OPERATIONS ON IODINE EXCRETION<sup>1</sup>

By J. P. GEMMELL AND W. F. PERRY

## Abstract

Further investigations have been carried out on the previously reported increase in iodine excretion which occurs following the stress of surgical operations. By means of adrenocorticotrophic hormone it was demonstrated in four subjects that increased activity of the adrenal cortex does not of itself cause an increase in iodine excretion. In six patients subjected to elective surgical procedures it was shown with the aid of radioactive iodine that the inorganic iodide of the body tissues is not the source of increased iodine excretion which follows such operations. Other possible causes and sources of the postoperative increase in iodine excretion are discussed.

## Introduction

In a previous communication (3) it was demonstrated that the stress of surgical operations in healthy human subjects, besides being associated with increased adrenal cortical activity, indicated by elevated urinary excretion of corticosteroids, was also accompanied by a very marked and sudden increase in the excretion of iodine in the urine.

Whether this alteration in iodine metabolism was a result of the extra production of cortical hormones or alternatively was an unassociated stress phenomenon was not established. In addition, although several possible sources of the extra urinary iodine were postulated its actual origin remained unknown.

The purpose of the present communication is to report observations concerning these points.

## Subjects

The subjects of these investigations are detailed in Table I. As in the previous series, all those on whom surgery was performed were essentially normal healthy people who were hospitalized for elective surgical procedures. On the basis of the usual clinical and laboratory criteria, none of the subjects were considered to have any thyroid or adrenal dysfunction.

## Methods

The analytical procedures for urinary constituents have been previously described (3). In order to examine the effect of increased adrenal cortical activity on iodine excretion in the absence of operative trauma, the adrenal cortex was stimulated by means of its trophic hormone (ACTH-Armour). This was administered intramuscularly to four patients in 25 mgm. amounts three to four times daily for from two and a half to five days.

<sup>1</sup> Manuscript received March 13, 1950.

Contribution from the Department of Medicine and the Department of Physiology and Medical Research of The University of Manitoba and from the Winnipeg General Hospital, Winnipeg, Canada.

TABLE I  
SUBJECTS OF EXPERIMENTS

Subject	Age	Sex	Diagnosis or operative procedure
1	45	M	Normal
2	43	M	Dermatitis
3	28	M	Disseminated sclerosis
4	42	M	Rheumatoid arthritis
5	41	M	Plate to radius and ulna
6	22	M	Excision of metatarsals
7	47	M	Arthrodesis; foot
8	25	M	Herniotomy
9	27	M	Herniotomy
10	56	M	Herniotomy
11	21	M	Normal
12	24	M	Normal
13	39	M	Normal
14	20	M	Normal

Concerning the origin of extra iodine excreted postoperatively it was decided to investigate the inorganic iodine of the body tissues as a possible source. This objective was attained by means of the following procedure.

A tracer dose of radioactive iodine as NaI<sup>131</sup> was administered and two hours allowed for diffusion throughout the body fluids in order to label the inorganic iodide of the tissues with radioiodine. The ratio of the concentration of isotopic iodine (% dose per 100 ml.) to that of stable iodine ( $\mu\text{gm.}$  per 100 ml.) was then determined in either plasma or urine or both. This ratio was determined before submitting subjects to operation and two to four hours after operation, at which time it has been observed that the plasma and urine levels of iodine are elevated. If the source of the postoperative iodine increase were from the labelled source, i.e., the inorganic body iodine, there should be no great change in the ratio following operation. On the other hand, if the extra iodine were from nonlabelled sources there should be a marked alteration of the ratio.

This measurement was performed in six patients who were subjected to operative trauma, five of whom showed postoperative increases in iodine excretion, and in four other persons who were not subjected to operation.

### Results

The results of ACTH administration in four subjects are shown in Fig. 1 together with, for comparison, the mean results reported for surgical trauma (3). It will be seen that increased levels of adrenal cortical hormones do not in themselves lead to an increased excretion of iodine in the urine.

In Table II is shown the ratio of isotopic to stable iodine in plasma or urine before and at two to four hours after operation together with the urinary iodine (I<sup>127</sup>) excretion during the 24 hr. before operation and during the first 24 hr. following operation.

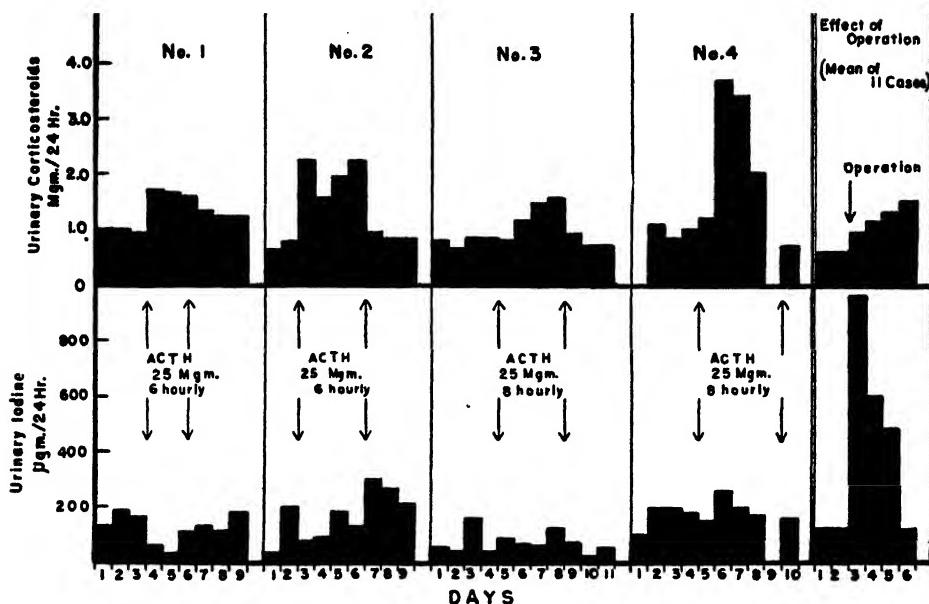


FIG. 1. Effect of ACTH on iodine excretion.

TABLE II  
INFLUENCE OF OPERATION ON IODINE METABOLISM

Subject	Procedure	Urinary $I^{131}$ , μg.m./24 hr.		Ratio $I^{131}/I^{127}$ in plasma or urine		
		Before operation	After operation	Before procedure'	2-4 hr. after procedure	% Initial value
5	Operation	45	907	0.38	0.02	4.0
6	Operation	145	105	0.19	0.13	69.0
7	Operation	118	697	0.34	0.01	3.5
8	Operation	156	475	1.10	0.23	20.0
9	Operation	116	2080	0.42	0.02	4.5
10	Operation	206	600	1.50	0.05	3.2
11	I.v. iodide	—	—	3.20	0.01	0.2
12	I.v. iodide	—	—	0.46	0.02	3.3
13	Nil	—	—	0.70	0.30	43.0
14	Nil	—	—	0.38	0.32	85.0

It will be seen that in six operative cases examined five showed a marked diminution of the ratio  $I^{131}/I^{127}$  following operation. The one exception was a subject who was also exceptional in not showing a postoperative increase in iodine excretion.

In contrast are two control subjects in whom no procedures were carried out other than determination of the ratio  $I^{131}/I^{127}$  at similar time intervals as in the above six subjects. In these two normal subjects the ratio declined

only slightly in comparison with that in the operated subjects. The decline of the ratio which occurs in the normal subject is presumably due to the gradual influx into the iodide compartment of the body of unlabelled iodine from dietary and other sources—e.g. degradation of thyroid hormone. In the traumatized subject this influx from other sources is greatly accelerated.

Also shown in Table II is the result of introducing suddenly into the circulation (by means of intravenous injection) 5 mgm. of unlabelled iodide. This was done in two subjects. The results duplicated the effects of operation in that there was an increase in plasma and urinary iodine but a decline in the ratio  $I^{131}/I^{127}$  following the injection.

### Discussion

From the data presented above it seems clear that the alteration in iodine metabolism which follows the stress of surgical operations is not due to the increased activity of the adrenal cortex and consequent increased production of cortical hormones which also takes place as a result of trauma. It would also appear that the postoperative production of iodine is from some source other than the inorganic iodide of the body tissues, i.e., from some unlabelled source, otherwise the ratio  $I^{131}/I^{127}$  would have exhibited only a slight decline similar to that observed in the normal subjects.

It is conceivable that the post-traumatic increase in iodine excretion is a result of an increased secretion of thyrotrophic hormone mobilizing iodine from the thyroid gland and also possibly a result of an accelerated breakdown of thyroid hormone in the peripheral tissues.

It has been shown by various investigations (2, 4) using histological and radioiodine techniques that the stress of cold brings about an increase in thyroid activity due to an elaboration of thyrotrophin. In addition Williams *et al.* (5) have induced thyroid hyperactivity by means of other stress situations as well as cold and at the same time have found evidence which indicates that stress is followed by an increased degradation of thyroid hormone peripherally. Further support of this latter concept is perhaps found in the elevation in metabolic rate which occurs following operations (1). Whether the post-operative increase in iodine excretion is a consequence of thyrotrophin secretion or a consequence of an accelerated breakdown of thyroid hormone, or both, remains to be investigated.

### Acknowledgments

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# THE FREE-CELL PHENOMENON IN ISOHAEMAGGLUTINATION<sup>1</sup>

BY KENNETH W. MCKERNs AND ORVILLE F. DENSTEDT

## Abstract

An hypothesis is presented to account for the invariable persistence of unagglutinated red blood cells in agglutinated samples even when potent isoagglutinating serum is used. It is postulated that the cells which remain free represent the few which become completely saturated with the agglutinin during the sensitization phase of the reaction. These cells, which amount to only a fraction of a per cent of the total originally present in the sample, are incapable of reacting with one another but can undergo agglutination with fresh cells of the same blood type thus indicating that they are morphologically normal. Evidence is presented in support of the hypothesis. The free-cell count is reproducible provided the agglutinating serum is potent and the conditions of the experiment are standardized. The influence of various factors on the free-cell count, and the usefulness of the count for following changes in the avidity of isoagglutinating serum with time, are discussed.

When erythrocytes are exposed to a specific agglutinating antiserum a few cells invariably escape agglutination even with highly potent sera. These so-called "free" or "unagglutinable" cells amount to a fraction of a per cent of the total number in the sample. The free-cell count may be reproduced with accuracy provided potent antiserum is used and the conditions such as the temperature, size, and shape of the container, rate of agitation, and others are kept constant. The failure of some of the cells to be agglutinated is of no consequence in qualitative tests such as blood-typing but, in certain quantitative procedures, the free-cell count may constitute the basis of the method. It is necessary in such cases to eliminate the possibility of variation due to changes in the conditions of the test. For example, in the Ashby method of differential agglutination for following the rate of elimination of the donor's cells from the recipient's circulation after transfusion, the free-cell count of the recipient's blood must be determined prior to the transfusion, and the conditions of agglutination must be standardized throughout the investigation.

Among the factors which strongly influence the free-cell count is the strength of the agglutinating serum (10). The strength or "potency" usually is expressed in terms of the "titer" and the "avidity". The former represents the maximum dilution of the serum that will produce specific and visible agglutination of a cell suspension or a colloidally dispersed antigen. However, it affords only a relative indication of the actual concentration of antibody since the value obtained will vary depending on the antigen content of the cells used, the duration of the test, the electrolyte concentration, temperature,

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Contribution from the Department of Biochemistry, McGill University, Montreal, Que.

This study represents part of a dissertation presented by K. W. McKerns in September 1949 in partial fulfilment of the requirement for the degree of Doctor of Philosophy at McGill University. A review of the study was presented at the meeting of the Committee on Blood and Blood Derivatives of the National Research Council in Washington, D.C. on July 8, 1949. The principal points of the study were reported also at the annual convention of the Canadian Physiological Society in Montreal on October 15, 1949.

and the rate of collision and intimacy of contact of the cells. It is well known that when the estimation is carried out in the test tube with centrifugation the titer value obtained may be from 6 to 10 times that given by the slide method in which the cells are brought in contact with one another by simple agitation. The relative nature of the titer is brought out in DeGowin's study in which titer determinations were made on a number of given isoagglutinating sera in several laboratories (5). The titer method, while adequate for testing the acceptability of antisera for routine blood-typing, is not entirely satisfactory for the quantitative evaluation of the antibody concentration in isoagglutinating sera.

The term "avidity" also is an indefinite one which implies the rapidity of action of an antiserum. This property apparently has more to do with the sensitization phase than with the aggregation phase of the agglutination reaction. It represents the affinity of antibody for antigen and thus reflects the rate of combination of the two. Normal anti-A and -B sera, and heterologous immune agglutinating sera such as anti-M and -N, when fresh, usually exhibit strong avidity. There appears to be no relation between this property and the titer. Certain antisera, notably anti-M and -N, may show strong avidity at a relatively low titer while, with certain others, the reverse relationship may exist. Pillemer (11) studied the two properties in various antisera and in isoagglutinin globulin fractions, and tested the effect of dilution, extraction of the lipid, and other treatments on the potency. He presents evidence that both a lipoprotein complex and a "heavy protein" are essential factors in the agglutinating complex, and that avidity is related to the lipoprotein. Studies on the assay of the potency of isoagglutinating sera have been reported also by Boyd (1), Oncley *et al.* (9) and others (8, 12). In these studies the estimation of potency is based on the titer, and on the avidity according to the respective times when agglutination becomes perceptible and when it reaches the maximum as judged by the appearance of the cell clumps.

In the study reported herein an effort has been made to characterize avidity on the basis of the free-cell count.

The reason why some of the red cells in any sample of blood fail to be agglutinated has received little systematic study. The only reports we have been able to find on the subject are those of Callender *et al.* (3) and of Young *et al.* (15). The former group found that the free-cell count is not influenced by the proportion of reticulocytes in the blood. The latter group of investigators showed that the reticulocytes are agglutinated in the same manner as mature cells and that the erythrocytes which fail to be agglutinated are not morphologically abnormal.

In the course of our study on the influence of various factors on the agglutination of erythrocytes an effort was made to ascertain the cause of the unagglutinable cell phenomenon. At first the following factors appeared to be among the plausible ones to be considered:

- (1) Failure of some of the cells to come in intimate contact with others.
- (2) Insufficiency of antibody to produce complete agglutination.
- (3) Absence of agglutinogen on the free cells, or perhaps, the presence of weakly reactive agglutinogen.

The first of these explanations is ruled out by the persistence of free cells even after repeated centrifuging of the sample and resuspension of the cell sediment. The second also can be eliminated by two observations, namely, (a) that the free-cell count cannot be reduced significantly by the further addition of antiserum, and (b), that when potent antiserum is used in the ordinary agglutination test there always is a sufficient excess of antibody to cause the agglutination of several further additions of cells.

The third explanation is more difficult to test, particularly with regard to the reactivity of the agglutinogens. However, it can be shown that the free cells are not devoid of agglutinogen, for, while they are unagglutinable among themselves, they are capable of reacting with fresh cells. The latter discovery shed new light on the problem and suggested a fourth explanation which is supported by a substantial body of evidence as set forth in the remainder of this paper.

The hypothesis to be described is based on the idea that the red cells may undergo various degrees of saturation with antibody. In accordance with present-day concepts the agglutinins are assumed to be at least divalent. In the initial, or sensitization phase of the agglutination reaction, agglutinin combines with a specific agglutinogen on the cells. In the second, or aggregation phase, the cells, on contact, become linked together by agglutinin and precipitate out in clumps. Both stages of the reaction appear to be specific (7). The hypothesis, to be presented, postulates that the great majority of the cells suffer collision and aggregation before sensitization is complete, while a few become completely saturated with antibody before making adequate contact with other cells. The saturated ones constitute the so-called free or unagglutinable cells. Since all their receptor sites are occupied these cells are incapable of undergoing aggregation among themselves. Another small proportion of the cells may approach saturation and subsequently either become saturated or undergo agglutination. Thus the free-cell count tends to fall exponentially to a virtually constant value on prolonged agitation or on repeated centrifugation of the sample.

From the foregoing hypothesis one may anticipate the following results:

- (1) that cell saturation should be favored by a high concentration of avid antibody. In other words, the free-cell count should be relatively high with a potent antiserum.
- (2) that a "zone" effect may be expected with highly potent antisera; in other words, optimal agglutination should be obtained only after appropriate dilution of the serum.
- (3) that the number of free cells should be greater when agglutination is carried out at higher temperatures, at least up to about 40° C.,

- (4) that the free cells should be capable of reacting with fresh, or incompletely saturated cells,
- (5) that the free cells obtained on agglutination at one temperature should be incapable of reacting with those formed at another temperature, since all should be saturated,
- (6) that it should be possible to demonstrate the presence of antibody on the free cells by means such as the Coombs test (4).

Confirmation of these inferences has been obtained.

## Experimental

### 1. Agglutination Procedure

A red-cell suspension is prepared by drawing blood up to the 0.5 mark in a white-cell pipette. The latter then is filled to the 11 mark with isotonic saline (0.9% sodium chloride) and the contents, after being mixed by agitation, are transferred to a small serological test tube. Into a dry white-cell pipette the cell suspension is drawn up to the 1.0 mark and the pipette then filled to the 11 mark with the temperature-equilibrated agglutinating serum.\* The contents are mixed and transferred to a serological tube which then is placed in a shaker and agitated at a constant rate (230 vibrations per minute\*\*), for 15 min. in a water bath maintained at a constant temperature (20° C.). The tube is centrifuged at 1500 r.p.m. in a table-model machine for two minutes. After the cell sediment is broken up and suspended by flicking the tube, the contents are again centrifuged. The tests are performed in duplicate and the free-cells counted in a hemocytometer. The counting is done with a medium objective and care is taken to avoid counting the white cells which may be present. The number of free cells per cu. mm. is obtained by multiplying the count taken over the entire field by the factor 269.

### 2. Reproducibility of the Free-cell Count

The number of free cells will vary with changes in the conditions of agglutination but, provided the conditions are kept constant, the count can be reproduced with accuracy. Table I illustrates the agreement obtainable under standardized conditions.

### 3. Variation in Free-Cell Count Over a Period of Time

Table II illustrates the variation of the free-cell count in fresh red-cell specimens removed periodically from three donors by finger puncture. The data on Specimen 1 were obtained in a previous study (10).

In Table III are given the free-cell counts on two lots of preserved red cells (whole blood). The specimens were preserved in citrate-glucose and kept at 5° C. Periodically samples were removed aseptically for the tests.

\* Prior to being used the serum is agitated for 30 min. in a water bath at the desired temperature.

\*\* A specially designed shaker is used, equipped with a Graham variable speed motor and a bath which may be set at various temperatures. The length of stroke of the shaker also may be adjusted.

TABLE I  
REPRODUCIBILITY OF FREE-CELL COUNT

Test No.	Specimen 1, in duplicate		Specimen 2, in duplicate	
	Cells: Group B	Serum: anti-B	Cells: Group B	Serum: anti-B
	Titer: 256	Dilution 1:1		Titer: 256
1	7250	6400	16,150	17,200
2	7000	6450	15,350	15,900
3	6200	8050		
4	7250	7550		
5	6700	7250		
6	6400	7800		
Mean 7025 ± 97 (S.E.)		Mean 16150 ± 525		

Tests on Specimen 1 carried out at 20° C.—agitated for 30 min. at 230 vibrations per min.  
 Tests on Specimen 2 carried out at 20° C.—agitated for 60 min. at 230 vibrations per min.  
 with two centrifugations.

TABLE II  
FREE-CELL COUNT ON FRESH RED CELLS

Day of test	Specimen 1	Specimen 2	Specimen 3
	Cells: Group A Serum: anti-A Titer: 5000 Diluted: 1:5	Cells: Group B Serum: anti-B Titer: 256 Diluted: 1:1	Cells: Group B Serum: anti-B Titer: 256 Diluted: 1:1
0	14,500		
1		22,600 } 22,375 22,150 }	16,600 } 16,250 15,900 }
3		10 a.m.: 10,200 } 10,350 10,500 }	6190 } 6350 6450 }
		3 p.m.: 9700 } 9825 9950 }	6750 } 6975 7200 }
7	11,000	15,600 } 15,075 14,550 }	12,100 } 12,750 13,400 }
10		14,250 } 14,775 15,300 }	10,700 } 10,200 9700 }
14	22,000		
18		6700 } 6575 6450 }	4300 } 4550 4800 }
21	23,500		
28		9400 } 9675 9950 }	8600 } 8475 8350 }
35	12,500		
43	16,000		
49	8000		

Anti-B serum, titer 256, diluted 1:1 with isotonic saline agitated 30 min. at 230 vibrations per min. and twice centrifuged and resuspended.

It will be observed that there was no difference in the magnitude of the variation in the free-cell count in the fresh and the preserved samples.

TABLE III  
FREE-CELL COUNT ON PRESERVED RED CELLS (10)

Day of test	Specimen 1	Specimen 2
	Cells: Group A Serum: anti-A Titer: 5000 Diluted 1:5	Cells: Group B Serum: anti-B Titer: 5000 Diluted 1:5
0	12,500	9000
2		12,500
5		11,500
7	19,000	11,000
9		6500
12		5500
14	11,000	
17		7500
19		9500
21	15,500	8000
23		10,500
26		9000
29		11,000
33		7000
35	15,000	
40		9500
43	11,000	
44		5000
47		10,500
49	31,000	
53		12,000
61		4500

It is evident that the free-cell count with given antisera and red cells may fluctuate to a considerable degree over a period of time. In a previous study (10) evidence was obtained that the fluctuations were attributable to a reversible type of change in the antisera rather than to an alteration in the cell agglutinogens. This is further confirmed by the results in the two experiments in Table II, in which the same antiserum was used concomitantly with the cells of two group-B individuals. The fact that the fluctuation in the free-cell counts in the two cases occurred at the same time and in the same sense again suggests that the antiserum, and not the cells, was the responsible factor.

#### 4. *Influence of Dilutions of the Serum on the Free-Cell Count*

With potent antisera the rate of aggregation usually is more rapid, and the size of the clumps tends to be larger in some of the diluted samples than with the undiluted serum. This frequently is seen in titer tests. To obtain the minimum free-cell count, it is necessary to dilute the serum. Obviously the minimum free-cell count affords a more reliable criterion of the completeness

of agglutination than does a judgment of the size of the clumps. Table IV illustrates the fall in the free-cell counts on dilution of various high-titer anti-A sera with isotonic saline.

TABLE IV  
INFLUENCE OF DILUTION OF THE SERUM ON THE FREE-CELL COUNT  
(Free-cell counts per cu. mm. of various dilutions of the sera)

Serum sample	Titer	Series of dilutions						
		Undiluted	1:1	1:2	1:5	1:10	1:20	1:40
1	2048	98,400				80,500	16,100	
2	2048				62,100	59,600	50,500	
3	512			22,000			7800	
4	2048			70,200			22,400	
5	1024		23,400				13,700	10,750

The same Group A cells were used in all the tests.

### 5. Free-cell Count after Repeated Addition of Fresh Cells

The following experiment illustrates the tendency of the free-cell count to remain practically unchanged when repeated agglutinations are carried out with a given amount of a potent antiserum, i.e., with a large excess of antibody initially present, and on repeated addition of red cells.

#### Procedure

A suspension of red cells (ANRh) was prepared by drawing whole blood up to the 0.5 mark in a white-cell pipette and then filling the latter to the 11 mark with isotonic saline. After shaking the pipette the contents were transferred to a small serological tube. With the same pipette (washed well and dried before each use) the cell suspension was drawn up to the 1.0 mark, and transferred to each of three serological tubes, A, B, and C. A fourth aliquot of suspension was pipetted to the 1.0 mark and the pipette then filled to the 11 mark with the agglutinating serum. The thoroughly mixed contents were transferred to a serological tube, D (kept at 25° C.) and then placed in the shaker for agglutination in the manner previously described. In the meantime the cells in Tubes A, B, and C were centrifuged for two minutes at about 1500 r.p.m. and the supernatant fluid was then removed carefully with a capillary tube. The three tubes, containing the sedimented cells, were placed in a water bath at 25° C. for temperature equilibration. After the prescribed agglutination period the agglutinated cells in Tube D were permitted to settle for two or three seconds and the supernatant fluid containing the free cells was transferred to Tube A\*. The cells were suspended by flicking the tube and then placed on the shaker for agglutination. After the usual period,

\* In some of the experiments the pipette was filled from the supernatant portions of duplicate agglutinated samples in order to increase the accuracy in making the transfer of the free cells.

the free cells from A were transferred to Tube B and the agglutination repeated. In a similar manner the free cells from B were added to C. By this procedure the volume of the samples and the conditions of treatment were kept nearly constant during the four runs. The experiment was repeated several times using different antisera.

TABLE V

CONSTANCY OF THE FREE-CELL COUNT ON REPEATED AGGLOMERATION OF CELLS WITH A GIVEN VOLUME OF POTENT ANTISERUM

Experiment	Antiserum			Free-cell counts per cu. mm. in the series				
	Type	Titer	Dil'n	I (1 vol. cells)	II (2 vol.)	III (3 vol.)	IV (4 vol.)	Mean
1	Anti-N	12	Undil'd	12,900	12,700	14,000	13,300	13,325 $\pm$ 298
2	Anti-A	512	"	6200		6650		6425 $\pm$ 225
3	Anti-B	128	"	10,200		10,100		10,150 $\pm$ 50
4	Anti-M	64	"	17,200		18,300		17,750 $\pm$ 550
5	Anti-N	64	"	12,100		13,400	14,800	13,433 $\pm$ 729
6	Anti-A ( $\alpha$ )	256	1 : 5	33,600		40,000		36,800 $\pm$ 3200
7	Anti-A ( $\alpha_1$ )	128	1 : 1	11,050		12,100		11,575 $\pm$ 525

The results of typical experiments are given in Table V. In Experiments 2 to 7 the free-cell count in the initial run was determined in the usual manner, i.e. the supernatant fluid containing the free cells was added to two volumes of sedimented normal cells. The mixture was agglutinated in the usual way.

Thus, when a large excess of antibody is present, the free-cell count remains remarkably constant on successive additions of fresh cells. It is evident that most of the free cells from each test must have undergone agglutination with fresh cells in the subsequent one. This was demonstrated further by labelling free cells with sulphhaemoglobin (7) and then adding them to a suspension of fresh cells. On examining the agglutinated cell sediment under the microscope the clumps were observed to consist of a mixture of labelled and unlabelled cells. A control test, using the sulphurated free cells alone with the supernatant fluid from the previous agglutination, produced no agglutination.

#### 6. Influence of Temperature on the Free-cell Count

Any factor which influences the rate of the reaction between antigen and antibody should be expected to influence the incidence of cell saturation. It may be anticipated, therefore, that the free-cell count will tend to be greater at higher temperatures, at least up to about 40° C. Above the latter temperature the results may be expected to become erratic because of denaturation of the proteins in the cells and the serum.

Experiments were performed with various anti-A, -B, -M, and -N sera at temperatures ranging from 5° to 40° C. The samples were shaken for 20 min. at 230 vibrations per minute, twice centrifuged, and the cell sediments

69

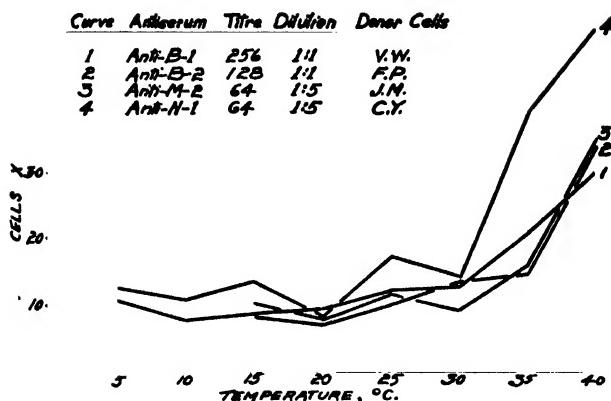


FIG. 1. Influence of temperature on the free-cell count obtained with avid isoagglutinating sera.

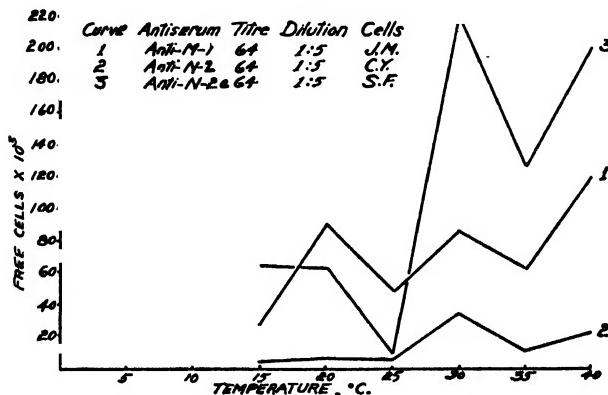


FIG. 2. Influence of relatively weak avidity on the free-cell count obtained at various temperatures.

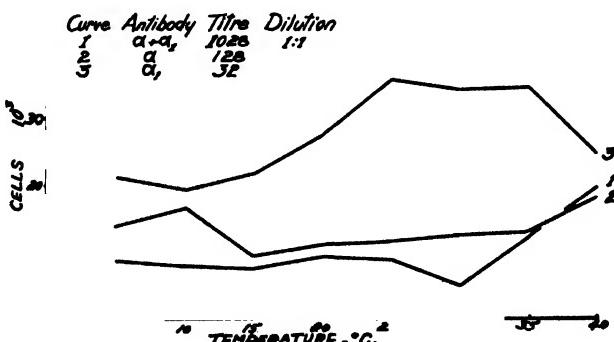


FIG. 3. Influence of temperature on the free-cell count obtained with the  $\alpha$  and  $\alpha_1$  isoagglutinins before and after separation.

resuspended after each centrifugation. The free-cell counts obtained are indicated by the curves in Figs. 1, 2, and 3.

The comparatively regular curves in Fig. 1 represent the free-cell counts obtained at various temperatures with sera of relatively high avidity. The less regular curves in Fig. 2 are typical of those with less avid sera (10, 11). The particular anti-M and anti-N sera used in the latter tests had been in storage for several months. Furthermore, it was necessary to dilute them to ensure a sufficient supply for the experiment.

Changes in temperature between 5° and 30° C. appear to have a relatively slight effect on the free-cell count with avid anti-A, -B, -M, and -N sera. Between 30° C. and 40° C., however, the count tends to be considerably increased and the size of the clumps smaller with increase in the temperature. These observations suggest that the cohesion of the aggregated cells may be weaker at the higher temperatures and that some of the cells may be shaken free from the clumps and perhaps may become saturated.

In view of the composite nature of anti-A sera, one might anticipate a peculiar type of behavior of the free-cell count with these sera depending on the concentration and avidity of the  $\alpha$  and  $\alpha_1$  or  $\alpha_2$  antibody constituents. Fig. 3 illustrates the free-cell counts obtained at various temperatures with a potent anti-A serum and with the  $\alpha$  and  $\alpha_1$  components after separation (see footnote p. 162). The curve with the  $\alpha_1$  agglutinin suggests that this antibody is relatively sensitive to changes in temperature. The poor recovery of the antibody in the process of isolation accounts for the low titer.

### 7. Reactivity of Free Cells Formed at Different Temperatures

It is to be anticipated that the free cells formed at different temperatures should not undergo agglutination when mixed since presumably all are saturated. This inference was confirmed in several experiments of which the data given in Table VI are representative.

TABLE VI  
MIXTURE OF FREE CELLS FORMED AT DIFFERENT TEMPERATURES

Sample	Temperature used in agglutination test	Free cells per cu. mm.		
		Experiment 1	Experiment 2	Experiment 3
(a)	20° C.	6450	5370	6450
(b)	30° C.	12,900	25,200	29,000
(c) Found	20° C.	8870	15,100	16,700
Expected		9675	15,285	17,850

The sample (c), consisting of equal volumes (the usual quantities) of the supernatants (containing the free cells) from (a) and (b), in each experiment was shaken at 20° C. Thus the observed free-cell count was close to the expected count, namely half the sum of the counts in (a) and (b).

In Experiment 1 cells of Group ANRh were used; in Experiment 2, Group ONRh, and in 3, ANRh. The same anti-N serum (titer 64, diluted 1 : 5) was used in the three experiments.

That the free cells are not devoid of reactivity was demonstrated in another experiment in which the supernatant serum, containing the free cells from one agglutination test at 30° C., was mixed with the prescribed quantity of fresh cells, and the mixture agglutinated at 20° C. The new free-cell count was compared with that in a sample which had been agglutinated at 20° C.

#### Procedure

Two lots of cells (ANRh) were prepared for agglutination in the usual manner and were agitated at 230 vibrations per minute for 50 min., one of the samples (*a*), at 30° C. and the other (*b*), at 20° C. In either case the specimen, after the period of agitation, was centrifuged for two minutes at 1500 r.p.m. The cells were resuspended, the tube again centrifuged, and the cells resuspended. After determining the free-cell count the supernatant (containing the free cells) from Specimen *a*, was added to the sedimented fresh cells (saline having been removed) in a sample (*c*) and the suspension agglutinated at 20° C.

The free-cell counts in the three samples are given in Table VII.

TABLE VII

FREE CELLS OBTAINED AT 30° C. AGGLUTINATED WITH FRESH CELLS AT 20° C.\*

Sample	Temperature of test	Free cells per cu. mm.		
		Series (1)	Series (2)	Series (3)
( <i>a</i> )	30° C.	23,600	19,400	29,000
( <i>b</i> )	20° C.	10,500	8600	6450
( <i>c</i> )	20° C.	10,500	9400	6700

\* Cells ANRh; serum anti-N, titer 64, diluted 1 : 5.

In Series 1, red cells of Group ANRh were used with anti-N serum (titer 64, diluted 1 : 5), and in series 2, the same donor's cells with anti-A ( $\alpha$ ) serum\* (titer 64, diluted two times). The agreement between the counts in (*b*) and (*c*) is close and indicates clearly that the free cells from the agglutination at 30° C. must have reacted with the fresh cells in the subsequent test.

To test whether any alteration is produced in the reactivity of the cells or the serum at 40° C. the red cells and the serum separately were heated at 40° C. for 30 min. On repeating the agglutination test with these materials

\* The serum was prepared according to the procedure of Landsteiner and Miller (13). Anti-A ( $\alpha$  and  $\alpha_1$ ) serum was absorbed repeatedly with Group A<sub>2</sub> cells until no further reaction was obtained with the cells. The agglutinated cells from the successive trials were suspended in a small volume of isotonic saline and warmed at 56° C. for two minutes. The sample then was centrifuged for two minutes at the latter temperature and the supernatant fluid removed. The titer of the released  $\alpha$  agglutinin was found to be 64.

at 20° C. the free-cell count was found to agree with that obtained with unheated cells and serum. At higher temperatures, however, the results tend to become erratic.

#### 8. Reaction of Saturated Cells with a Different Antibody

The question arose whether cells, after saturation with one antibody, are capable of reacting with a different antibody. The following experiments indicate that a second and perhaps a third reaction can occur.

Red cells of Group AMNRh were agglutinated at 20° C. with anti-M\* in one tube, and with anti-N\* in another. The free-cell counts in the two samples were respectively 32,250 and 15,100 per cu. mm. The contents of the two tubes then were mixed, twice centrifuged, resuspended, and examined. The free-cell count was found to be reduced to 3225 per cu. mm.

In another experiment cells of Group ANRh, after agglutination separately with anti-A serum, and anti-N respectively, gave a free cell count of 13,450 and 24,310 respectively per cu. mm. On agglutination with mixed anti-A and anti-N sera the free-cell count was found to be 7260 per cu. mm.

These observations confirm the evidence obtained in another phase of our study, namely, that the different agglutinogens appear to be distributed in patches on the red cell surface and that the saturation of one antigen with the corresponding antibody appears not to interfere greatly with the reactivity of the other antigens.

#### 9. Estimation of Avidity of Antisera

##### a. Characterization of Antisera

The following three criteria have been used in our study for evaluating the potency of antisera:

- (1) Titer, with, and without, centrifugation.
- (2) Free-cell count under constant conditions of agglutination.
- (3) The free-cell count at specified times during the period of the test.

The first of these affords a rough index of the antibody concentration; the second indicates the number of saturated cells which, in turn, reflects the avidity of the serum, i.e. the rate of combination of antigen with antibody; the third also reflects the rate of sensitization and thus the avidity. With avid sera, saturation of the cells occurs rapidly, and the free-cell count tends to reach a constant value within a relatively short time. Thus the rapidity with which the count reaches a constant level, rather than the magnitude of the final count, is the important criterion in the test.

To obtain a comparable base of reference for each serum the dilution was chosen which gave the lowest free-cell count. This was determined by the following procedure using a 15 min. period of shaking since, with our apparatus, the reaction is virtually complete within 15 min.

\* The anti-M serum previously had been absorbed with Group-N cells and the anti-N with -M cells.

### Procedure

Red cells, containing the appropriate agglutinogen, were agglutinated with the antiserum by shaking the sample for 15 min. at 20° C. at 230 vibrations per minute. The tubes then were centrifuged twice at 1500 r.p.m., each time for two minutes. The clumps were broken up after each centrifugation by sharply flicking the tube. This procedure was repeated for the various dilutions in the titer series and the dilution which gave the lowest free-cell count was noted. A quantity of the original serum, sufficient for the final test, was prepared in this dilution.

In the avidity test three samples of cells and diluted antiserum, which may be designated (a), (b), and (c) respectively, were prepared in the usual way and were treated in the following manner:

- (a) Sample shaken for one minute at 20° C. and then centrifuged for one minute.
- (b) Sample shaken as in (a) but with two centrifugings of two minutes duration, the cell sediment being resuspended after each centrifugation.
- (c) Sample shaken for 15 min. at 20° C., followed by centrifuging twice for two minutes and resuspension of the cells after each centrifugation.

The free-cell counts obtained in several experiments of this kind are given in Table VIII.

TABLE VIII  
FREE-CELL COUNTS AFTER THREE TREATMENTS AT 20° C.

Antiserum	Titer	Serum dilution used	Free-cell counts per cu. mm.		
			Sample (a)	Sample (b)	Sample (c)
Anti-A ( $\alpha$ and $\alpha_1$ )	2048	1 : 1	26,600	12,600	6100
Anti-B	256	1 : 1	14,250	10,500	10,200
Anti-M	64	1 : 5	54,000	11,200	6700
Anti-N	64	1 : 5	15,900	6300	6200
Anti-A ( $\alpha$ )	128	1 : 5	22,600	14,300	10,700
Anti-A ( $\alpha_1$ )	64	1 : 4	22,600	38,000	29,600

It will be observed that the free-cell count reached a constant value most rapidly with the anti-B and the anti-N sera thus indicating that these were the most avid of the series. The anti-M serum was a sample which had been stored in the cold for several months. With the  $\alpha_1$  serum the free-cell count was higher in (b) than in (a) owing to disintegration of the clumps on continued agitation. Thus an increase in the count may occur if the cells in the aggregates are very weakly bound together; a simple flicking of the tube in some instances, e.g. in Rh agglutination, may release individual cells from the aggregates. While this serum showed a low avidity, the behavior is not a characteristic of anti-A sera as a rule.

*b. Decrease in Avidity of Antiserum with Time*

The avidity of serum may undergo a gradual decrease during storage particularly if the material has been repeatedly frozen and thawed. The deterioration may be accelerated by unfavorable conditions of storage. Table IX illustrates the fall in avidity of one anti-A serum during storage at 5° C. The original titer of the serum was 2048 (at 20° C. tested with red cells of type ANRh), and appeared to be unchanged at the 25th day of storage when tested with the same cells. However, a slight decrease was detected in the titer of the  $\alpha_1$  agglutinin in special tests.

TABLE IX  
DECREASE IN AVIDITY OF AN ANTI-A SERUM WITH STORAGE

Dilution of serum	Free-cell count per cu. mm.	
	With serum when fresh	Serum after storage 25 days
1 : 2	72,300	8600
1 : 20	22,400	10,200
1 : 40	10,750	12,600

Shaking period 15 min., followed by two centrifugations

It will be observed that the free-cell count with the fresh serum was high in the 1 : 2 dilution and fell to 10,750 in the 1 : 40 dilution. After storage for 25 days the number of saturated cells in the 1 : 2 dilution was comparable with that of the 1 : 40 dilution of the fresh material. The counts in the 1 : 20 and 1 : 40 dilutions in the 25-day specimen represent an incomplete reaction.

### Discussion

In our study, instead of evaluating the avidity of isoagglutinating sera on the basis of the respective times when agglutination becomes perceptible and when it reaches the maximum as judged by the appearance of the clumps, we have based our estimate on the free-cell count. The criteria used were: (a) the degree of dilution of the serum necessary to give the lowest free-cell count, and (b) the free-cell count at the end of 15 min., by which time the count reaches a practically constant value under the condition of the test. By both the older procedure and ours, in order to obtain reproducible results it is necessary to standardize the various conditions of the tests such as the volume of the sample, the proportion of cells and antiserum, temperature, and the rate and mode of agitation. Because of the variability of the results with the conditions, the values obtained have a relative rather than an absolute significance, and thus, the methods are of limited value for purposes of standardization. The free-cell method offers the advantage that the values are based on an accurate count rather than on the judgment of the worker. Furthermore, the ability to define the conditions of the procedure

makes possible the duplication of the results in any laboratory. The chief value of the method, however, lies in its usefulness for following changes in the avidity of antisera during storage and other treatments, and in studying the antigen content of the red cell. Its usefulness will be extended when more is known about the nature and absolute concentration of the isoagglutinins in the serum and the agglutinogens in the cells.

The saturation concept appears to account for the free-cell phenomenon and certain others which hitherto have not been satisfactorily explained. Also, it fits in with current ideas on the mechanism of agglutination whether the process be viewed from the standpoint of the law of mass action, or as an adsorption phenomenon (14, 2). Further studies on the kinetics of the process would be facilitated by a clearer understanding of the chemical basis of avidity and the mode of distribution of the various antigens on the cell surface. There is little evidence that the reaction between agglutinogen and agglutinin is reversible to any considerable extent at temperatures below 40° C. At considerably higher temperatures in the region where denaturation of the cell and plasma proteins occurs, a partial release of antibody can be effected.

The observed day-to-day fluctuation in the free-cell count with given red cells and potent agglutinating sera has not yet been satisfactorily explained. It has been shown that the free-cell count, when carried out under controlled conditions, is reproducible within about 5-8%, depending on the cells and the antiserum, provided the tests are performed about the same time, i.e. on the same day. When repeated at intervals over a period of several days or weeks, on the other hand, the count may undergo a fairly large day-to-day variation which apparently is not due to loss of potency. The observation that the degree of variation was found to be the same for fresh and for efficiently preserved red cells in concurrent tests on a given donor's blood, suggests that the cause lies in the antiserum and not in the cells. In a previous study (10) as well as in that reported here, we obtained evidence that the isoagglutinin, or some other influential component of the antiserum, is capable of undergoing a reversible type of change which appears to influence the avidity of the antibody. This behavior, furthermore, is accentuated when the antiserum is diluted, even though the material may still be of high titer and potency. An effort was made in our study to eliminate these changes by storing the sterile antiserum in small sealed tubes at 5° C., and equilibrating it at the time of use by agitating it in a water bath for 30 min. at the desired temperature. Despite these and other precautions the periodic variation in the free-cell count was found to persist.

The periodic fluctuation of the free-cell count of the recipient's blood does not seriously affect the usefulness of the Ashby method for following the elimination of the donor's cells after transfusion. During the early period of the experiment, when the total number of free cells may be from 200,000 to 500,000 per cu. mm. depending on the quantity of blood transfused (200 to 500 ml.), the variation in the recipient's free-cell count is of little significance.

Thereafter, and until the experiment is completed, the only effect of the variation is to produce a slightly irregular, rather than a smooth cell-elimination curve. Finally, when all the donor's cells have been eliminated, the curve levels off at the recipient's free-cell count. If the agglutinating serum is fairly fresh at the commencement of the tests, and is stored under sterile conditions at 5° C., it is unlikely that serious deterioration of the potency will occur (at least with anti-A or anti-B) during the four-month period of the experiment.

With the occasional highly potent anti-A agglutinating serum of the hyper-immune type it is almost impossible to obtain closely reproducible free-cell counts in successive agglutinations (as described on p. 159, Table V) even when the serum is suitably diluted. This observation leads one to suspect that unusual factors sometimes may be present, such as A-blocking antibodies the occurrence of which has been reported by Hubinont (6).

That the titer values obtained with a given isoagglutinating serum and the cells of various individuals of the same blood group or type may differ quite widely has long been known. This observation suggests that the concentration and distribution of the agglutinogen in question may be quite different from one blood to another. It is generally accepted, for example, that homozygous cells, e.g. AA, contain more of the antigen A and thus exhibit a stronger reaction towards anti-A than do the heterozygous AO cells. There are few individuals, if any, with the exception of identical twins, who are identical with respect to kind, content, and distribution of the red-cell agglutinogens, and thus it is understandable why the free-cell count with a given antiserum should differ with individual bloods. Since the free-cell count is partly a function of the agglutinogen content of the cells it is clear also why the count and the titer have little absolute significance for evaluating the antibody content of the serum.

An effort has been made to obtain information regarding the mode of distribution of the agglutinogens on the surface of the erythrocyte. However the different agglutinogens may be distributed, whether in a uniform pattern or mosaic, or in patches, it is significant that while the free-cells do not react to any considerable extent among themselves, the count will drop abruptly if a different antiserum be added, e.g. if after having determined the free-cell count with anti-A serum, anti-M be added. The apparent facility with which the second antibody reacts suggests that previous occupation of the A-sites with anti-A does not make the M-sites inaccessible to anti-M. A further report will be published on this phase of our study.

The number of free cells is relatively high with ordinary anti-Rh agglutinating sera which usually are not of high potency. Because of the weak cohesion between the cells in the aggregates many of the cells may become dispersed again on agitation of the sample. Owing to the unavailability of potent sera of this class it has not been possible for us to carry out a systematic study on the Rh-anti-Rh system.

### Acknowledgments

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We are grateful also to Dr. R. L. Denton, Director of the Red Cross Blood Transfusion Service for the Province of Quebec, for supplying specimens of potent agglutinating sera, and for helpful criticisms.

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## A STUDY OF HUMAN HYPERSENSITIVITY TO COMPOUNDS OF THE MUSTARD GAS TYPE<sup>1</sup>

By A. M. MOORE AND J. B. ROCKMAN

### Abstract

A method has been developed for expressing quantitatively the results of measurements of human skin sensitivity to compounds of the mustard gas type. Using this method a study has been made of induced hypersensitivity to *bis*( $\beta$ -chloroethyl)sulphide, and to ethyl *bis*( $\beta$ -chloroethyl)amine, and of cross reactions involving these two compounds. The relationship between hypersensitivity and the "flare reaction" has been investigated, and a simple method of inducing the latter is reported. A possible significance of these findings in connection with the healing of mustard gas burns is suggested.

Many simple chemical compounds are known to be capable of inducing hypersensitivity in man and in laboratory animals. The extensive work of Landsteiner and his associates (10-14) using citraconic anhydride and picryl chloride, and the work of other investigators (7, 8, 17) with various aromatic nitro compounds has clearly indicated that a property common to such chemical allergens is their ability to react with tissue proteins with the formation of conjugates which behave somewhat like synthetic antigens in that they exhibit a specificity determined by the prosthetic group.

The present paper contains the results of experiments, using volunteer human subjects, in which the sensitizing agents employed have been two compounds of the mustard gas type, viz., *bis*( $\beta$ -chloroethyl)sulphide, and ethyl *bis*( $\beta$ -chloroethyl)amine. That the former of these can react with certain proteins was clearly indicated in the work of Berenblum and Wormall in 1939 (2), and has been amply verified in more recent studies (1, 5, 6, 9). It is perhaps not surprising, therefore, that it should have a sensitizing action on human skin when suitably applied. There are several reports on this subject in the literature of the past few years (2, 15). The extreme potency of this type of compound as an allergen is, however, somewhat surprising, and indeed, it must be regarded as an appropriate tool for studying the phenomena connected with hypersensitivity.

The experiments described in the ensuing pages of this report are arranged in four sections. The first deals with the normal range of sensitivity to mustard gas and the effect upon it of various types of exposure to this compound. Part 2 contains the results of similar experiments with one of the "nitrogen mustards". In the third part the question of cross sensitivity toward the two agents is considered. The final section deals with the phenomenon of reactivation, here termed the "flare reaction".

In the following, the abbreviation MG (mustard gas) is used for *bis*( $\beta$ -chloroethyl)sulphide, and NM (nitrogen mustard) for ethyl *bis*( $\beta$ -chloroethyl)amine.

<sup>1</sup> Manuscript received May 2, 1950.

Contribution from Chemical Warfare Laboratories, Ottawa. (Now, Defence Research Chemical Laboratories.)

The word "sensitivity" is used in the special sense of skin sensitivity as measured by the technique described herein, and the term "normal" is used to denote men who had not previously been exposed to either MG or NM.

### Methods

The method used in all the sensitivity tests was as follows. Serial dilutions of the compound being tested, in low-boiling petroleum ether, were prepared covering the desired range of concentrations. A single drop of each dilution was allowed to fall freely on to the volar surface of the forearm of the subject from a 27 gauge hypodermic needle, bevel up, on a syringe held at an angle of 45 degrees to the horizontal. This method gave a fairly uniform drop size which was estimated to be 4.50 cu. mm. with a standard deviation of 0.22 cu. mm. The arm was then observed after 24, 48, and 72 hr., and the number of visible reactions recorded. The highest dilution giving a visible reaction during the 72 hr. period was taken as the measure of sensitivity.

If the percentage of men reacting to each dilution is plotted against the logarithm of the concentration, a sigmoid curve is obtained similar to the action curves of many pharmacological agents. Bliss (3, 4) has shown how to transform such data into a linear relationship for convenience in analysis. The procedure he describes has been applied to the data to calculate the regression constants and also the concentration to which 50% of the population react, a quantity which might be termed the "median effective concentration" and which, in the following, is represented by the symbol EC 50.

No attempt was made at the time of application to prevent evaporation of the solute, but it may be assumed that the amount evaporating was a nearly constant fraction of the amount applied, particularly since the area over which the drop spread was approximately the same in all cases. The sensitivity end points are therefore expressed in arbitrary units which, in this case, correspond to the amount of vesicant applied. No estimate has been attempted of the actual amount of penetrated MG or NM required to produce a reaction in the skin.

The MG and NM used in these experiments were carefully purified preparations supplied by the organic chemistry section of Chemical Warfare Laboratories, Ottawa.

### Experimental

#### *Part 1—Sensitivity to MG*

##### *Group 1—Normal Sensitivity to MG*

A group of 60 normal men was tested, as described above, with dilutions of MG in petroleum ether ranging from 1 : 1000 to 1 : 8000 by volume. Forty-five of these were also tested with a dilution of 1 : 500. In this test, as in all the others, the relative positions of the various dilutions being used were randomized with the aid of a table of random numbers. This was done in an attempt to eliminate possible errors due to systematic differences in sensitivity of the skin in different regions of the forearm. The results are given in Table I.

TABLE I  
SENSITIVITY TO MG

Log of MG concn.*	Group 1		Group 2		Group 3	
	No. of men	% reacting	No. of men	% reacting	No. of men	% reacting
3.301	45	100	17	88	—	—
3.000	60	90	17	82	14	100
2.699	60	57	17	53	24	96
2.398	60	15	17	47	24	92
2.097	60	0	17	12	24	75
1.796	—	—	17	6	33	54
1.495	—	—	—	—	33	27
1.194	—	—	—	—	33	9
0.893	—	—	—	—	33	3

\* Concentration expressed in cu. mm. MG per liter of solution.

*Group 2—Sensitivity to MG after a Single Burn*

Seventeen men were given minor burns by an application of 0.0020 ml. of a 10% (by volume) solution of MG in benzene, delivered by means of a micrometer syringe to each of three spots on the same forearm. (The three burns may be regarded as equivalent to a single larger burn.) Two weeks later test dilutions of MG in petroleum ether were applied to the opposite forearm. The results are summarized in Table I.

*Group 3—Sensitivity to MG after Two Successive MG Burns (One Week Interval)*

A group of normal men was given three simultaneous burns with 0.0020 ml. of a 10% (by volume) solution of MG in benzene applied to one forearm. Seven days later a second set of three burns was produced by the same means on the same forearm, but at different sites. Seven days after the second set of burns the sensitivity of the men to MG was tested; the results are given in Table I.

In Table III are summarized the results of the probit analysis. The significance of the differences in EC 50 between the foregoing groups has been examined by means of the usual "t" test, using as the criterion of significance  $P = 0.05$ . On this basis the difference in EC 50 between Groups 1 and 2 is not significant, whereas the corresponding difference between Groups 1 and 3 is. A similar test has been applied to the regression coefficient " $b$ ". Both the differences 1-2 and 1-3 are significant.

Thus it appears that, under the conditions of this experiment, a single MG burn followed by a two week rest period did not result in any appreciable general sensitization of the skin, whereas two similar MG burns seven days apart followed by a seven day rest period produced a definite degree of hypersensitivity.

**Part 2—Sensitivity to NM**

Experiments similar to those described in Part 1 have been carried out with NM. In all cases the solutions of the free base were freshly prepared just before the tests were made.

**Group 4—Normal Sensitivity to NM**

A group of normal men was selected for the test. Drops of serial dilutions of NM in petroleum ether were applied, by the technique described under "Methods", to the volar surface of the forearm of each man. The percentage of men reacting to each dilution within the 72 hr. observation period was recorded. The results are given in Table II.

TABLE II  
SENSITIVITY TO NM

Log of NM concn.*	Group 4		Group 5	
	No. of men	% reacting	No. of men	% reacting
3.000	21	100	—	—
2.602	38	87	9	100
2.301	38	66	9	89
2.000	48	15	9	67
1.699	48	2	9	44
1.398	48	0	9	44
1.097	—	—	9	22

\* Concentration expressed in cu. mm. per 100 ml. of solution.

**Group 5—Sensitivity to NM after two NM Burns (One Week Interval)**

The procedure in this case was similar to that for Group 3 but using NM instead of MG. The burns were produced by the application of 2.0 mgm. pure NM to each of three spots on the forearm. The interval between the first and second sets of burns was seven days. Seven days after the second set of burns was induced the sensitivity was tested on the opposite forearm in the usual way.

Examination of the results of the probit analysis, as summarized in Table III, indicates that the differences in EC 50 and in slope between Groups 4 and 5 are both significant ( $P = 0.05$ ).

**Part 3—Cross Sensitivity**

Attempts were made to determine whether any cross sensitivity could be demonstrated in the case of men sensitized either with MG or NM. The general procedure was to give a group of men two sets of burns, at an interval of seven days, with one compound, and then, seven days after the second burns, to test their sensitivity with the other compound. In certain experiments sensitivity tests with one or both compounds were made before the

first burns were applied. The results of all these tests are summarized in Table III together with those of the foregoing groups for convenience in comparison.

TABLE III  
SUMMARY OF RESULTS OF TESTS WITH MG AND NM

Group No.	No. of men tested	Description of group	EC 50	S.E. of EC 50	Slope of regression line (b)	S.E. of (b)	D.F.
			Log units				
1	45-60	Normal sens. to MG	2.665	0.049	4.014	0.444	2
2	17	Sens. to MG after one MG burn	2.531	0.079	1.878	0.326	2
3	14-33	Sens. to MG after two MG burns	1.777	0.052	2.150	0.241	3
4	21-48	Normal sens. to NM	2.251	0.040	3.692	0.450	1
5	9	Sens. to NM after two NM burns	1.632	0.139	1.455	0.512	2
6	22	Sens. to MG after two NM burns (not previously tested)	2.602	0.081	1.776	0.283	3
7	15	Sens. to MG after two NM burns (previously tested with MG)	2.301	0.093	1.599	0.327	3
8	18	Sens. to MG after two MG burns (previously tested with NM)	1.502	0.117	1.031	0.187	5
9	26	Sens. to NM after two MG burns (not previously tested)	2.225	0.163	2.830	0.488	1
10	18	Sens. to NM after two MG burns (previously tested with NM)	1.824	0.078	1.781	0.312	3

S.E. = Standard error.

D.F. = Degrees of freedom.

EC 50 = Concentration to which 50% of group react (calculated) (arbitrary concentration units).

The ordinate and abscissa for the regression line are "probits" and "log of concentration" respectively.

From the standpoint of cross sensitivity certain of the differences between the tabulated values of EC 50 are of interest. Tests of significance have been applied to these and to the slopes of the calculated regression lines.

The differences in EC 50 between Groups 1 and 6, and between Groups 4 and 9 are not significant. That is to say, two burns with MG, one week apart, did not sensitize these men to NM, nor did two NM burns, one week apart, sensitize them to MG.

The difference between the EC 50's of Groups 6 and 7 is a borderline case but may be regarded as probably significant. This would imply that a simple sensitivity test to MG, consisting of an application of four drops of dilute solutions of MG in petroleum ether, can, at least when coupled with two NM

burns, produce a moderate degree of sensitization to MG. Whether the NM burns had any effect here can not be decided.

The difference in slope between the regression lines for Groups 1 and 6 is significant according to the usual criterion.

Groups 3 and 8 do not differ sufficiently in their EC 50's to meet the requirements of  $P = 0.05$ , but the difference does satisfy  $P = 0.10$ . Furthermore, the difference in slopes between Groups 3 and 8 is clearly significant. It would appear therefore, that in Group 8 the small applications of NM used for the sensitivity test may possibly have augmented the sensitivity to MG while, at the same time, increasing the variability of response of the men.

Groups 9 and 10 do not differ significantly either in slope or in EC 50.

#### *Part 4—The Flare Reaction*

In the course of a separate series of experiments involving MG burns, cases of "flare reaction" were occasionally observed. By this expression is meant the sudden recrudescence of a healed lesion or an abrupt increase in the severity of a relatively minor lesion after a lengthy period of quiescence. In many of these cases the original lesions had merely been erythema which had faded and returned apparently to normal only to break out again with severe vesication after a lapse of a week or longer.

An examination of the data on the observed cases indicated that, in every one, the men had been exposed at least twice, and that the flare occurred shortly following a second or later exposure. Although considerable numbers of men had been exposed more than once the number of flares was relatively small. The data were therefore examined in the hope of finding some factor connected with the exposures which was common to, and characteristic of the flare cases.

The only factor which appeared to bear on the subject was the interval between the repeated burns. The examples of flares occurred only in those experiments in which the interval between the burns was about one week or longer. In view of this fact it seemed distinctly probable that the flare reaction was a hypersensitivity phenomenon, and that the significance of the interval between the burns was related to the incubation period required to develop sensitivity. If this were true there were probably other ways in which the hypersensitivity might disclose itself, even when flares did not occur.

It was decided to carry out repeated exposures to MG in an attempt to elicit flare reactions and, at the same time, to measure the skin sensitivity of the men before and after the two burns.

The men used in this study are included in the groups described in the preceding sections. The MG burns were produced by three simultaneous applications of 0.0020 ml. of a 10% (by volume) solution of MG in benzene to one forearm. The second set of burns was produced similarly after an interval of seven days. The two sets of burns were usually, but not invariably, located on the same arm. Variation in this respect did not make any apparent difference in the results.

The lesions were examined daily, and their measurements, together with a visual estimate of their severity, were recorded daily until they were healed.

In a series of 71 men who were given two sets of MG burns in this way, 19 cases of flares involving the sites of the primary lesions were observed. These ranged in severity from simple reactivation, with vesication, of a healed lesion to severe erythema and oedema involving the entire arm, and, in a smaller number of cases, to generalized dermatitis.

It may be remarked in passing that similar data have been obtained with NM. Twenty-four men exposed twice to subvesicant doses of NM at an interval of seven days yielded six cases of flares.

The time of development of the flare varied between one and nine days after the second burn, the average time being four and a half days for the 19 cases of flares induced by MG. In Table IV are given the results of MG sensitivity tests for 18 men who exhibited flare reactions following a second MG burn.

TABLE IV  
SENSITIVITY TO MG OF MEN WHO SHOWED FLARE REACTIONS

Log of MG concentration	No. of men tested	% of men reacting
2.097	18	100
1.796	18	89
1.495	18	78
1.194	18	50
0.893	18	33
0.592	18	5

\* Concentration in cu. mm. per liter of solution.

The calculated EC 50 for this group, in log units, was 1.177 with a standard error 0.059. This figure is significantly less than the EC 50 for Group 3. Indeed this group had the lowest EC 50 (i.e., highest sensitivity) of any of the groups. Thus there appears to be a definite association between the occurrence of the flare reaction and the possession of a high degree of sensitivity to MG.

### Discussion

The sensitivity of men to either MG or NM, as measured by the technique illustrated in this paper, is clearly altered by burns induced by MG or NM respectively. Two burns at an interval of a week have a much more marked effect than a single burn, although even a single, small application, such as is involved in a sensitivity test, appears to have some effect after a rest period of about two weeks (Cf. Groups 6 and 7).

No evidence of simple cross sensitivity has been found. Men who had become hypersensitive to MG were still normal toward NM provided that they had not had previous contact with NM. Likewise, men hypersensitive to

NM were normal to MG if they had not had a prior exposure to MG. However, there is some indication that sensitization to MG may be enhanced by simultaneous sensitization to NM (Cf. Groups 3 and 8).

The flare reaction is of particular interest. In our experiments about a quarter of the men who were exposed twice, at an interval of a week, to a small dose of either MG or NM developed the reaction in varying degree. In the MG experiments the initial burns were in all cases quite superficial, indeed in many cases there was merely an erythematous response. The second set of burns was applied at a time when, according to the results of Group 2, there was no general hypersensitivity. Nevertheless this second application, even when made on the opposite arm elicited in certain individuals a second and more severe response at the site of the first lesions. It would appear that although no general sensitivity is present, the actual site of the primary burn is in fact hypersensitive at this time. It was not possible to devise a suitable technique to test this suggestion directly, but the evidence of the flare experiments points strongly in this direction. If it may be assumed that the site of a primary MG lesion becomes hypersensitive after about 11 days—the average time of occurrence of the flares—the possibility suggests itself that such hypersensitivity might be a contributing factor to the notably slow rate of healing of MG burns, particularly in view of the evidence that MG residues are fixed in the skin at the site of application (16).

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## **NOTICE**

The Canadian Journal of Research is at present published in six sections, A to F. Starting with January 1, 1951, these sections will be published as separate journals under distinctive names and the designation Canadian Journal of Research will no longer be used. The present names and the corresponding new names are as follows:

<b>PRESENT NAME</b>	<b>NEW NAME</b>
Canadian Journal of Research, Section A (Physical Sciences)	Canadian Journal of Physics
Canadian Journal of Research, Section B (Chemical Sciences)	Canadian Journal of Chemistry
Canadian Journal of Research, Section C (Botanical Sciences)	Canadian Journal of Botany
Canadian Journal of Research, Section D (Zoological Sciences)	Canadian Journal of Zoology
Canadian Journal of Research, Section E (Medical Sciences)	Canadian Journal of Medical Sciences
Canadian Journal of Research, Section F (Technological Sciences)	Canadian Journal of Technology

In order to preserve continuity the present sequence of volume numbers will be retained, and in each case the volume for 1951 will be Volume 29.

The subscription rates for the Journals will remain as at present.



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## ANTIBIOTICS PRODUCED BY MICROCOCCI AND STREPTOCOCCI THAT SHOW SELECTIVE INHIBITION WITHIN THE GENUS *STREPTOCOCCUS*<sup>1</sup>

By R. G. E. MURRAY<sup>2</sup> AND L. J. LOEB

### Abstract

Two strains of *Micrococcus epidermidis* and five of *Micrococcus pyogenes* were found to produce antibiotics that were detected by using a mucoid *Streptococcus pyogenes* as test organism. The antibiotics were also active against some other Gram-positive organisms, but not against Gram-negative bacteria. The *M. epidermidis* strains inhibited most  $\beta$ -haemolytic streptococci with the exception of mucoid Lancefield Group C. The *M. pyogenes* strains were active against the majority of mucoid *S. pyogenes* (Group A), but few or none of the nonmucoid strains were inhibited. Streptococci of Groups B, C, and G were not affected. On the basis of antibacterial spectrum and characteristics of the antibiotics (dialysis, stability, and production curves) the seven strains were divided into four groups producing antibiotics of different types.

Three strains of  $\beta$ -haemolytic streptococci were tested which were known to produce antibiotics. Two of these showed a selective inhibition similar to the micrococci. They inhibited all of the mucoid *S. pyogenes* but few of the non-mucoid; they inhibited all strains of Group C, both mucoid and nonmucoid, but did not inhibit the Group G strains tested.

These observations suggest that, apart from capsulation, there may be a fundamental metabolic difference between the majority of mucoid and nonmucoid strains of *S. pyogenes*.

### Introduction

While testing strains of *Micrococcus pyogenes* for the production of hyaluronidase by a streak-plate method, Murray and Pearce (15) noted that a small number of strains inhibited the mucoid *Streptococcus pyogenes* used as indicator organism. To determine whether this interference could be avoided, other mucoid *S. pyogenes* were tried and most of these were antagonized by the inhibitory micrococci. It was also noted that stock nonmucoid strains were not inhibited. This preliminary observation led to a more detailed study of the inhibitory strains. The possibility was kept in mind that selective antibiotics might direct attention to metabolic systems that distinguish closely related streptococci.

A similar situation arose when Sherwood (20) was testing  $\beta$ -haemolytic streptococci for hyaluronidase production by the same technique. A number of strains were found, representing nine Lancefield Groups, that inhibited the

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test organism and certain other streptococci. Three of these strains, kindly provided by Dr. Sherwood, have been included in this study because of the similarity of the method of selection.

### Methods

The micrococci were surveyed by a streak method on blood agar plates. The test organism was the mucoid strain of *S. pyogenes* (M. Strep. 9) used by Murray and Pearce (15). Active *Micrococcus* strains and the *Streptococcus* strains obtained from Dr. Sherwood were tested in the same way against a variety of organisms to determine their antibacterial spectra. The plates were incubated aerobically. During the course of the work the effect of incubation anaerobically or under increased carbon dioxide tension was investigated. (There was no change in the results except that one *Micrococcus* (3064) was not active in the carbon dioxide atmosphere.) This testing procedure on blood agar largely excluded the possibility that activity might be due to peroxides.

A cell-free fluid containing the antibiotic was obtained from all but one of the active strains by a variation of a "cellophane sac" technique described by Heatly and Florey (9). Cellophane tubing was suspended from a plugged tube in the stopper of a side arm vacuum flask and autoclaved. This sac was filled with sterile broth and the inoculum was distributed over the outer surface of the cellophane *via* the side arm of the flask. The apparatus was incubated at 37° C. and the inhibitory action of the broth was tested at intervals during a 14 day period.

Activity was determined qualitatively by using standard glass cylinders or filter paper discs ( $\frac{1}{2}$  in.) on blood agar plates seeded with the test organism. Quantitative estimations were made by serial halving dilutions in dextrose broth for a rough estimate, and by harmonic dilutions for a more accurate titration. An inoculum of 0.1 ml. of a 24-hr. dextrose-broth culture of the test organism in a total volume of 5 ml. was used for each dilution. Each series was incubated for 18 to 24 hr. at 37° C. The end point of a titration was read as the highest dilution of antibiotic showing complete absence of growth.

### Media

The *blood agar* used for the survey was that used by Murray and Pearce (15). The *broth* used for antibiotic production in the cellophane sac consisted of:

Ficin digest of meat*	400 ml.
Proteose peptone #2 (Difco)	5 gm.
Proteose peptone #3 (Difco)	5 gm.
Distilled water to 1 liter	pH 7.0

\* Similar to the papain digest medium of Asheshov (1) but substituting ficin for papain.

The dextrose broth used for titration of activity consisted of:

Nutrient broth, dehydrated (Difco)	8 gm.
Proteose peptone #2 (Difco)	5 gm.
Proteose peptone #3 (Difco)	5 gm.
Dextrose	12 gm.
Distilled water to 1 liter	pH 7.4

## Results

### MICROCOCCAL ANTIBIOTICS

#### *The Incidence of Inhibitory Micrococci*

Five of 158 coagulase-positive micrococci and two of 47 coagulase-negative micrococci showed antibiotic activity against M. Strep. 9. The five inhibitory *M. pyogenes* strains (1831, 1648, 4457, L8400, 3064) were isolated from human secretions. One of the coagulase-negative organisms (2921) originated from a sample of cream pie while the other (CI) was isolated as a plate contaminant; they were probably derived from human sources and they correspond closely to *M. epidermidis*.

#### *Range of Activity of Inhibitory Micrococci*

Active strains found in the initial screening were further tested, by the streak method, against a variety of organisms. The effect on various species of *Streptococcus* is summarized in Table I, and on some other Gram-positive

TABLE I  
SUSCEPTIBILITY OF STRAINS OF *Streptococcus* TO INHIBITORY MICROCOCCI

Microorganisms surveyed	No. of strains tested	Number of strains inhibited by:					
		1831	1648	4457	L8400	3064	2921 or CI
Beta-haemolytic streptococci							
Lancefield Group A nonmucoid	23	1	2	2	0	0	22
Lancefield Group A mucoid	8	6	7	7	6	3	8
Lancefield Group B nonmucoid	6	0	0	0	0	0	4
Lancefield Group C nonmucoid	6	0	0	0	0	0	5
Lancefield Group C mucoid	6	0	0	0	0	0	0
Lancefield Group G nonmucoid	6	0	0	0	0	0	6
Alpha-haemolytic ( <i>viridans</i> ) streptococci	8	2	3	3	2	3	3

organisms in Table II. The two *M. epidermidis* strains gave identical results. None of the active strains inhibited any of the Gram-negative bacteria that were tested: *Escherichia coli*, *Salmonella typhosa*, *Salmonella schottmuelleri*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Proteus* sp., *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Neisseria catarrhalis*.

TABLE II

## SUSCEPTIBILITY OF VARIOUS GRAM-POSITIVE ORGANISMS TO INHIBITORY MICROCOCCI

Microorganisms surveyed	No. of strains tested	Number of strains inhibited by:					
		1831	1648	4457	L8400	3064	2921 or CI
<i>Micrococcus pyogenes</i>	13	4	11	11	0	7	6
<i>Micrococcus</i> sp. coagulase-negative	4	1	1	1	0	0	1
<i>Diplococcus pneumoniae</i> (various types)	9	9	9	9	2	1	7
<i>Corynebacterium diphtheriae</i>	6	6	6	6	0	6	0
<i>Bacillus</i> sp.	4	2	3	3	0	4	0

The range of activity shown in the streak tests allowed preliminary classification of the antibiotics produced. The activity of the *M. epidermidis* strains was clearly differentiated from the others and showed a more general activity against the streptococci and no action against *Corynebacterium diphtheriae*. Three of the *M. pyogenes* strains (1831, 1648, and 4457) seemed to be almost identical; strain 3064, although somewhat resembling these, was later differentiated by another property (see Table III). L8400 was distinct owing to its narrow range of activity.

The selective inhibition of certain streptococci is illustrated in Table I. The *M. pyogenes* strains were active against few or none of the nonmucoid Lancefield Group A streptococci, whereas they inhibited most of the mucoid strains of the same group. On the other hand these strains inhibited none of the streptococci of Lancefield Groups B, C, and G. The *M. epidermidis* strains inhibited the majority of the streptococci tested, excepting mucoid Group C.

In addition to M. Strep. 9 another strain of Group A type 9 mucoid *S. pyogenes* and seven nonmucoid type 9 strains were available. The mucoid strains were sensitive to all of the inhibitory *M. pyogenes* except 3064, while none of the nonmucoid strains were sensitive. All were inhibited by *M. epidermidis* 2921. A similar result was obtained with a series of Group A type 6 cultures.

Attempts to obtain stable mucoid variants of the nonmucoid Lancefield Group A *S. pyogenes* strains were unsuccessful, but on two occasions non-mucoid variants of M. Strep. 9 were obtained. Both of these variants were inhibited by the same strains of *Micrococcus* and to the same degree as the parent strain.

*Characteristics of the Inhibitory Substances*

All the inhibitory strains except 3064 produced inhibitory substances that diffused through cellophane. This was tested by growing the micrococci on the surface of a piece of cellophane placed on a blood agar plate. The cellophane and growth was removed after overnight incubation and the area so

exposed was seeded with a sensitive organism and incubated to find out whether diffusion had taken place. The cellophane sac technique for obtaining active, cell-free broth could be applied to the organisms producing dialyzable antibiotics. The broth inside the sacs was tested daily by the assay cylinder method and halving dilution titration. Strain 2921 showed a steady production of antibiotic up to the eighth day (Fig. 1); the activity of this broth has

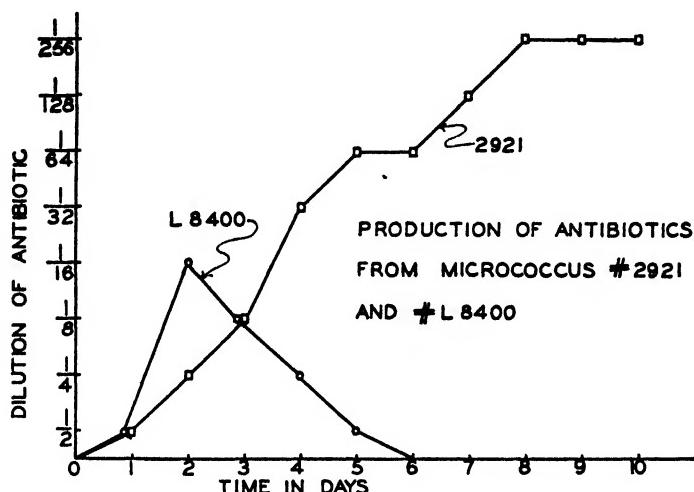


FIG. 1. A graph showing the titers of antibiotic obtained during production in cellophane sacs using strains 2921 and L 8400.

remained constant for 18 months at 4° C. Strains 1831, 1648, 4457, and L8400 showed greatest inhibition at two – five days, after which the activity declined (Figs. 1 and 2). L8400 had an earlier peak. No satisfactory means was found for producing the antibiotic from 3064.

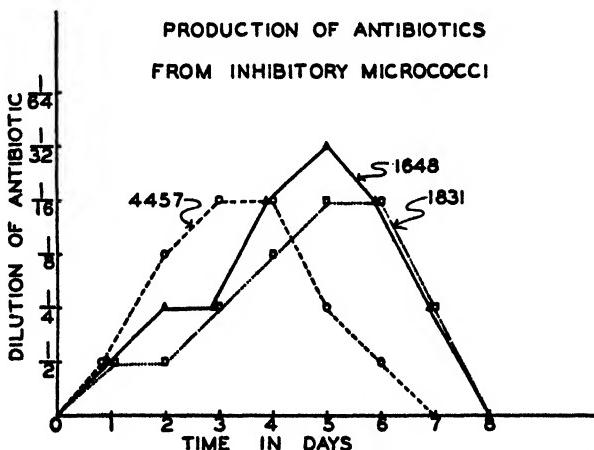


FIG. 2. A graph showing the titers of antibiotic obtained during production in cellophane sacs using strains 4457, 1648, and 1831.

Bacteriostatic or bactericidal action was determined in the tubes of an antibiotic titration. Tubes showing inhibition after overnight incubation were diluted with broth and reincubated to detect growth. The mode of action of the inhibitory micrococci is bacteriostatic, although two times the minimum inhibitory dose of the antibiotic produced by *Micrococcus* 2921 seemed to have bactericidal action towards M. Strep. 9. The action of 3064 is unknown.

Table III summarizes the characteristics of the inhibitory micrococci. The organisms are arranged in four groups on the basis of the coagulase test and properties of the antibiotic substances. The antibacterial spectra gave the same general classification.

TABLE III  
SOME CHARACTERISTICS OF ANTIBIOTIC-PRODUCING MICROCOCCI

	Coagulase-positive strains: <i>M. pyogenes</i>		Coagulase-negative strains: <i>M. epidermidis</i>	
	Group			
	I	II	III	IV
Strains	1831 1648 4457	L8400	3064	2921 CI
Incidence	$\frac{3}{158}$	$\frac{1}{158}$	$\frac{1}{158}$	$\frac{2}{47}$
Hyaluronidase	+	-	+	-
Pigment	Golden	Faint golden	Golden	White
Antibiotic characteristics				
Dialysis	+	+	-	+
Stability	-	-	-	+
Peak of production in days	4 - 5	2	?	8

#### STREPTOCOCCAL ANTIBIOTICS

As has already been mentioned, Sherwood *et al.* (20) isolated a number of streptococci that inhibited other *Streptococcus* strains. They used the same test organism (M. Strep. 9) that was used in this study.

Three active strains (one representative from each of Lancefield Groups A, D, and H) were obtained from Sherwood. Their activity against selected organisms is shown in Table IV. The streptococcal and micrococcal antibiotics were similar in the overall spectrum of activity. Two strains (Groups

TABLE IV

## SUSCEPTIBILITY OF VARIOUS GRAM-POSITIVE ORGANISMS TO INHIBITORY STREPTOCOCCI

Organisms surveyed	No. of strains tested	Number of strains inhibited by:		
		C203 Lance. A	AND Lance. D	"H" Lance. H
<i>Streptococcus</i>				
Lancefield Group A, mucoid	9	9	9	9
Lancefield Group A, nonmucoid	21	3	3	21
Lancefield Group B, nonmucoid	1	1	1	1
Lancefield Group C, mucoid	6	6	6	6
Lancefield Group C, nonmucoid	4	4	4	4
Lancefield Group G, nonmucoid	5	0	0	3
<i>Micrococcus pyogenes</i>	10	0	0	0
<i>Corynebacterium diphtheriae</i>	5	0	0	5

A and D) inhibited all the mucoid Group A strains and relatively few of the nonmucoid strains, while the Lancefield Group H strain showed a more general inhibition. The three streptococci inhibited all the Group C strains. Data for *M. pyogenes* and *C. diphtheriae* are provided for comparison with the antagonistic micrococci.

## Discussion

The production of inhibitory substances by *M. pyogenes* and other micrococci is not a new observation. De Freudenreich (7), Doeble (3), and Löde (13) described the inhibitory action of occasional strains. Schiotz (17) used a strain of *M. pyogenes* for the treatment of diphtheria carriers, an application that was revived by Dulescouet and Ballet (6) and by Lesbre and Merle (12). Dujardin-Beaumetz (4) isolated an *M. pyogenes* active against Gram-positive organisms and an unidentified *Micrococcus* (5) that inhibited the growth of a very wide range of bacteria including Gram-negative organisms. Prica (16) reported a strain of *M. pyogenes* active against species of *Klebsiella*. The above studies give little information other than the range of species inhibited as a basis for comparison. A few recent studies are more detailed. Jennings and Sharp (10) reported the isolation of a number of strains of *M. pyogenes* whose antibacterial spectrum was confined to Gram-positive bacteria. One of these organisms was investigated in detail by Gardner (8) who isolated a nondialyzable antibiotic and described its characteristics. Su (21) has also given a detailed description of "micrococcin", an antibiotic produced by a *Micrococcus* isolated from sewage. Most of the inhibitory strains are active against various Gram-positive and inactive against Gram-negative organisms as are those reported here. Two exceptions are noted in the strains described by Dujardin-Beaumetz (5) and by Prica (16); the former inhibited a notably wide range of Gram-negative organisms.

Two of the four groups (Table III) of antagonistic micrococci we have isolated show similarities to some of the strains reported in the literature. The three strains of Group I correspond to the *M. pyogenes* isolated by Dule-scout and Ballet (6) and Lesbre and Merle (12). The *Micrococcus* 3064 (Group III) is similar to the *M. pyogenes* strains described by Dujardin-Beaumetz (4) and by Gardner (8). The antibiotics of Groups II and IV do not seem to have counterparts reported in the literature.

The *M. epidermidis* strains inhibited the majority of  $\beta$ -haemolytic streptococci tested, but did not inhibit the mucoid Group C strains. The *M. pyogenes* strains, differing in their specificity, were active against a high proportion of mucoid Group A streptococci, a very few nonmucoid Group A and not against any representatives of Groups B, C, or G. This indicates not only some specificity as to group, but also selection of a distinct morphological variant within the group. For all these antibiotics, it is the susceptibility of the mucoid variation that is distinguished. In one case, mucoid Group A are largely susceptible; in the other case, mucoid Group C are notably resistant. Since the two mucoid organisms have a hyaluronic acid capsule in common (11, 14, 18, 19), it is tempting to speculate that the antibiosis is dependent upon some metabolic system associated with capsulation. However, with each antibiotic, if either one of the mucoid groups is inhibited the other is not. Therefore, the apparent association of susceptibility with the mucoid state and the hyaluronic acid capsule is probably fortuitous.

The antibiotic-producing streptococci discovered by Sherwood (20) resemble the micrococci in that their activity is confined to Gram-positive organisms. Two of the three strains of inhibitory streptococci that were tested also showed a selective inhibition, within Group A, of the mucoid strains (Table IV). In fact, these are rather more selective than were the micrococci since all the mucoid strains were inhibited. A further contrast was that both mucoid and nonmucoid Group C strains were inhibited.

It is generally believed that most antibiotics owe their activity to competitive inhibition of enzyme systems. Assuming that the selective antibiotics described in this paper act in this fashion, it is possible that the specificities observed may indicate variations in physiological mechanisms within the  $\beta$ -haemolytic streptococci. These streptococci exhibit considerable variations in pathogenicity and host specificity that correlate well with the immunological grouping. If the assumption is true that the antibiotics act upon specific enzyme systems, the selection of mucoid Group A strains may be interpreted in two ways: (1) The mucoid mutation may involve some more fundamental enzyme system than that needed for capsule production, or (2) the mucoid mutation is most often associated with one physiological kind of *S. pyogenes*, which may be independent of immunological type. Of these two hypotheses, the latter fits the facts more closely. Most of the mucoid strains but only a few of the nonmucoid strains were susceptible. Further, nonmucoid mutants of a susceptible mucoid strain had the same sensitivity as the parent. A similar sort of experimental approach is not possible with

our Group C representatives, because the mucoid strains are *Streptococcus zooepidemicus* ("animal pyogenes") and the nonmucoid are *Streptococcus equisimilis* ("human C"), which have other distinguishing properties (2).

Such arguments lend color to the possibility that selective antibiotics may direct attention to new and worthwhile attacks upon the physiology of the  $\beta$ -haemolytic streptococci and *S. pyogenes* in particular.

Another aspect of the inhibitory micrococci may be the role they could play in the ecology of skin and mucous membranes. This activity could occur only in occasional situations, since active strains form only about three per cent of isolates.

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# THE CHEMICAL ANALYSIS OF PURIFIED INFLUENZA VIRUS A (PR8 STRAIN) CONTAINING RADIOACTIVE PHOSPHORUS<sup>1</sup>

By A. F. GRAHAM

## Abstract

Purified influenza virus A (PR8 strain) was found to contain about 11% phospholipid and 5% nucleic acid in agreement with previously reported work. The method of Schmidt and Thannhauser, applied to the nucleic acid fraction of the virus, indicated the presence of 4.5% pentose nucleic acid and 0.3% desoxypentose nucleic acid. When influenza virus was grown in the allantoic membrane of the embryonated egg in the presence of inorganic radioactive phosphorus both phospholipid and nucleic acid components of the virus were found to contain the isotope. The specific radioactivity of the nucleic acid fraction was about four times that of the phospholipid.

## Introduction

In previous work (4, 5) it was shown that when the PR8 strain of influenza virus A was grown in the allantoic membrane of the embryonated egg in the presence of inorganic radioactive phosphate, the virus, when subsequently purified, contained a small amount of the isotope. There was no exchange of P<sup>32</sup> between the virus and inorganic phosphate and it was concluded that the virus had been labelled with the isotope during its growth in the cells.

Since it has been reported by Taylor (15) that the greater part of the phosphorus of the virus is contained in the phospholipid and nucleic acid it was of interest to determine whether the P<sup>32</sup> in the radioactive virus was also present in these components. If either or both of the phospholipid and nucleic acid were found to be labelled, it would afford additional evidence that the isotope was incorporated into the structure of the virus. The work described in this paper indicates that both phospholipid and nucleic acid were labelled.

Analyses of the nucleic acid content of influenza virus, using quantitative colorimetric tests for pentose and desoxypentose, have been reported by Taylor (15) and by Knight (7). While both pentose and desoxypentose nucleic acids seem to be present, there is still some doubt over their exact proportions, as pointed out by Beard (1). The present work indicates that these colorimetric tests for nucleic acids may be subject to interference from other constituents present in the virus particle and consequently an independent method, that of Schmidt and Thannhauser (11), has been applied in an attempt to determine the relative proportions of the two nucleic acids. From the results of these analyses it would appear that the PR8 strain of influenza virus A contains about 4.5% pentose nucleic acid and 0.3% desoxypentose nucleic acid.

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## Methods

### *Determination of Total Phosphorus*

The method has been fully described in a previous paper (5).

### *Determination of Radioactive Phosphorus*

All the radioactivity measurements were made on solutions of material in water or organic solvents. The method, which was also used throughout the previously reported work (4, 5), is described here in detail.

The radioactive solutions were pipetted onto 1½ in. diameter filter paper discs fixed to the tacky surface of a 2 in. wide strip of "Scotch" cellophane tape. After drying the discs under an infrared lamp a second strip of tape was pressed firmly over the top. An aliquot of 0.2 ml. of solution was sufficient to saturate the paper discs; where the radioactivity of the material was low, several 0.2 ml. aliquots were added to each disc, the paper being dried between additions. The cellophane tape was trimmed off close to the edge of the disc, leaving a "sandwich" of filter paper between two layers of tape. This was fixed in a brass mount and inserted under a Geiger-Müller counter tube of the end-window type. The brass mount was constructed in such a way that the radiation from an area of the filter paper exactly 1 in. in diameter registered on the counter.

A routine set of five radioactivity determinations was made with this method on an aqueous solution of P<sup>32</sup>, as inorganic phosphate, to determine its accuracy. With an average counting rate of 3450 impulses per min. (150 times background) the standard error of the mean was 1.1%. Inverting the paper discs under the counter had no measurable effect on the counting rate, nor did a total of three thicknesses of cellophane tape over the discs reduce the number of impulses registered.

As the method was used for estimating the radioactivity in biological fluids which contained appreciable amounts of protein, it was necessary to determine whether the presence of protein would interfere with the determination. Accordingly, a series of twofold dilutions of a solution of P<sup>32</sup>, as inorganic phosphate, was made in 2% haemoglobin. At the same time, a similar series of dilutions of the same solution was made in distilled water. Radioactivity determinations were made on each series of dilutions as described above. In both series, the activity decreased in direct ratio to the concentration of P<sup>32</sup>, the activities of corresponding dilutions in each series being the same within experimental error.

The method was calibrated against aqueous solutions of two uranium salts (C.P. grade) both of which had stood at least two years to ensure that they were in equilibrium. The  $\beta$ -ray disintegration rates of the uranium sources were calculated from data given by Kamen (6). This calibration was later checked against a RaD + E standard source from the National Bureau of Standards, Washington. A solution of P<sup>32</sup> calibrated against the Washington

standard contained 5330  $\mu$ rd. per ml. When measured against a uranium acetate solution it contained 5880  $\mu$ rd. per ml. and against a uranium nitrate solution 5950  $\mu$ rd. per ml.

In this paper, as in the previous report (5), the activities of  $P^{33}$  sources are expressed in counts per minute (c.p.m.); the results may be converted to microrutherfords using the factor 1000 c.p.m. as equivalent to a  $\beta$ -ray activity of 85.8  $\mu$ rd.

#### *Determination of Pentose Nucleic Acid (PNA) by the Orcinol Method*

PNA was estimated by the orcinol method according to the directions of McRary and Slattery (10). Two specimens of commercial yeast nucleic acid containing 7.90% P (Schwartz) and 7.85% P (Eimer and Amend) were used as standards. Although the green color developed in this test is due to the pentose in PNA the results were expressed in terms of the phosphorus content of the nucleic acid, that is, as pentose nucleic acid phosphorus (PNAP). When per cent transmittance, read at 660 m $\mu$  against reagent blanks, was plotted against PNAP for the two PNA specimens the curves were linear between 0-13  $\mu$ gm. PNAP and superimposable.

According to Schneider (12, 13) the nucleic acids of animal tissue may be completely extracted with 5% trichloroacetic acid (TCA) at 95° C. This method was applied to one of the specimens of PNA (Schwartz). The nucleic acid was extracted completely in 15 min., judged by phosphorus analysis, leaving a small residue presumably of protein. When the orcinol reaction was applied to a series of dilutions of the extract and the results plotted in terms of phosphorus content, the curve was identical with the two former curves.

Since the slopes of standard curves obtained in this test varied from one set of determinations to another owing to small variations in conditions of color development, a set of five dilutions of one of the standard PNA solutions was always run at the same time as an unknown solution. Thus the color density of the unknown solution could always be referred to a standard curve obtained under exactly the same conditions.

#### *Determination of Desoxypentose Nucleic Acid (DNA) by the Diphenylamine Reaction*

The diphenylamine reaction for DNA was carried out according to the directions of Seibert (14). Two specimens of calf thymus nucleic acid containing 7.32% P and 8.20% P were used as standards.\* When per cent transmittance at 600 m $\mu$  of the color developed in the diphenylamine reaction was plotted against phosphorus content for each of the two specimens, the two curves were superimposable and linear between 0-50  $\mu$ gm. DNAP. A weighed quantity of one of the specimens was extracted with 5% TCA at 95° C. The extract contained all the phosphorus of the original DNA and in the diphenylamine test gave a curve almost identical to the two former curves.

\* I am indebted to Dr. G. C. Butler for one of the specimens of calf thymus nucleic acid.

As in the orcinol test a set of five dilutions of a standard DNA solution was always run simultaneously with an unknown solution.

### *Preparation of Purified Influenza Virus*

Purified influenza virus was prepared by the method which has already been fully described (5).

### **Experimental**

#### **CHEMICAL ANALYSIS OF INFLUENZA VIRUS**

Before carrying out any analytical work on radioactive virus, it was necessary to determine the distribution of phosphorus between the virus constituents. For analysis, suspensions of purified influenza virus were prepared. An aliquot was taken for total phosphorus estimation and a further aliquot for dialysis against several changes of distilled water at 5° C. After 48 hr. the agglutinated virus was sedimented, dried *in vacuo* from the frozen state and then over phosphorus pentoxide in a vacuum desiccator.

The weighed dry material (10-25 mgm.) was stirred mechanically for 45 min. at room temperature with 4 ml. of alcohol-ether mixture (3/1, v./v.). The extraction was repeated on the sediment after centrifugation and the material was finally washed once with a small amount of ether and dried *in vacuo*. This alcohol-ether insoluble residue was considered to contain the protein and nucleic acid of the virus.

The combined alcohol-ether extracts were evaporated to dryness at 30° C. in a stream of nitrogen and the residue was extracted with several small portions of petroleum ether (b.p. 40°-60° C.) at room temperature. In accordance with the earlier work of Taylor (15) this extract was assumed to contain the phospholipid of the virus. Phosphorus and weight determinations were made on the various fractions.

The results of the fractionation are shown in Table I, the figures representing analyses on four different virus preparations. In each row of the table, the figures are averages of results obtained on at least three of the four preparations since all the determinations were not carried out on every preparation.

TABLE I  
CHEMICAL ANALYSIS OF INFLUENZA VIRUS A (PR8 STRAIN)

Fraction	Percentage of virus weight*	Phosphorus, % of virus weight*
Dried whole virus	100	0.96
Alcohol-ether soluble	26	0.51
Petroleum ether soluble	24	0.48
Petroleum ether insoluble	—	0.04
Alcohol-ether insoluble	76	0.45

\* Values are based on dry weight of virus.

The virus would appear to contain about 11% phospholipid, calculated from the phosphorus content of the petroleum ether soluble fraction. The results are in agreement with those of Taylor (15) for this virus.

#### *Fractionation of the Alcohol-Ether Insoluble Residue*

A strongly positive orcinol test for pentose was obtained with the alcohol-ether insoluble residue. While an unmistakably positive diphenylamine test for desoxypentose was not obtained on this fraction nor on whole virus, in our hands this has proved to be a relatively insensitive color reaction. Moreover the diphenylamine reaction is subject to interference by proteins and other substances as indicated by von Euler and Hahn (17).

Therefore, an attempt was made to separate and estimate the two nucleic acids of the virus by the method of Schmidt and Thannhauser (11). This procedure was tested out on a mixture of calf thymus nucleic acid, yeast nucleic acid, and crystalline egg albumen of known phosphorus contents, the recovery of the two nucleic acids being almost quantitative when present in roughly equal proportions. When the method was applied to the alcohol-ether insoluble residues of four different preparations of influenza virus 94.0%, 90.6%, 93.7%, and 93.5% of the total phosphorus of the residue appeared with the PNA fraction.

If phosphoprotein were present in the virus, its phosphorus would appear in the PNA fraction as inorganic phosphate. The procedure of Delory (3) was therefore applied to the separated PNA fraction, in two cases, to precipitate any inorganic phosphorus so that it could be estimated separately. In neither case was more than a trace of inorganic phosphorus found. It is well to mention, however, that the precipitation of inorganic phosphate was not quantitative when the amount of inorganic phosphorus present was less than 15  $\mu\text{gm}$ . per ml. To avoid this difficulty the precipitation method of Delory was applied to solutions containing known amounts of inorganic phosphate (0-30  $\mu\text{gm}$ . P per ml.) and a calibration curve prepared relating inorganic P added to that recovered from the precipitation. When a solution was made up containing 8.70  $\mu\text{gm}$ . P per ml. as inorganic phosphate and 5.55  $\mu\text{gm}$ . P per ml. as yeast nucleic acid phosphorus, using the precipitation method and calibration curve, the inorganic phosphate was estimated as 8.40  $\mu\text{gm}$ . P per ml.; the precipitation was carried out under the same conditions as obtained in the influenza virus experiments.

It would appear from these results that about 6% of the phosphorus of the alcohol-ether insoluble residue of the virus is present in DNA and about 94% in PNA. This would be equivalent to approximately 0.3% DNA and 4.5% PNA in the whole virus.

#### *Determination of PNA and DNA in the Virus by Colorimetric Tests*

Since the above results for PNA and DNA in the virus were quite different from previously reported results (7, 15) an attempt was made to determine the two nucleic acids by quantitative colorimetric tests.

Following separation of the two fractions from the alcohol-ether insoluble residue of the virus by the Schmidt and Thannhauser method, both orcinol and diphenylamine tests were carried out on the PNA fraction. It was observed that practically all the phosphorus could be extracted from the DNA fraction with 5% trichloroacetic acid for 30 min. at 90° C.; the diphenylamine test for DNA was carried out on this extract. The results are recorded in Table II. Since the orcinol and diphenylamine reactions were standardized

TABLE II

DISTRIBUTION OF PHOSPHORUS IN COMPONENTS OF ALCOHOL-ETHER INSOLUBLE RESIDUE OF INFLUENZA VIRUS SEPARATED BY SCHMIDT AND THANNAUSER METHOD

Fraction	Total phosphorus, $\mu\text{gm.}$	Phosphorus by orcinol test, $\mu\text{gm.}$	Phosphorus by diphenylamine test, $\mu\text{gm.}$
Pentose nucleic acid	62.8	53.8	7.7
Desoxypentose nucleic acid	4.3	—	2.6

in terms of the phosphorus contents of the respective nucleic acids as explained earlier, the figures in columns three and four of Table II give the amounts of phosphorus associated with these two acids in the virus. It is observed that the total amount of phosphorus determined from the colorimetric tests in this experiment checks fairly well with the total phosphorus estimated by direct determination. The PNA fraction separated by the Schmidt and Thannhauser method would appear to contain a small amount of DNA judged by the results of the diphenylamine test. The orcinol reaction was carried out on the DNA fraction but it was impossible to determine whether or not a slight green color had formed because of the presence of a brown pigment which appeared during color development. Similar results were obtained with a second preparation of virus.

It should be remarked that despite the reasonable agreement between the results of the two methods as shown in Table II, the colorimetric estimations came under suspicion for two reasons. Firstly, both ribose and yeast nucleic acid yielded a clear brilliant green color in the orcinol reaction whereas the influenza virus fractions often gave an olive green color with a tendency to slight opalescence. Secondly, the diphenylamine tests on the virus fractions were almost invariably slightly brown in color instead of the pure blue obtained with DNA in this reaction. Both these observations suggested the presence of substances in the virus which might interfere in the color tests.

Evidence that the colorimetric tests were not entirely trustworthy when applied to influenza virus fractions was obtained in a further experiment. The alcohol-ether insoluble residue from a purified virus preparation was treated for 30 min. with 5% trichloroacetic acid at 90° C. in an attempt to extract completely the nucleic acids. Eighty-eight per cent of the phosphorus

was removed by this treatment, 87% in a second such experiment. Both orcinol and diphenylamine tests were applied to the extract and to the extracted residue. The results are shown in Table III. It is observed that

TABLE III

DISTRIBUTION OF PHOSPHORUS IN HOT TRICHLOROACETIC ACID EXTRACT AND RESIDUE OF ALCOHOL-ETHER INSOLUBLE PORTION OF INFLUENZA VIRUS

Fraction	Total P, μgm.	P by orcinol test, μgm.	P by diphenylamine test, μgm.
Trichloroacetic acid extract	167.3	157	41.8
Extracted residue	23.3	37.5	12.0

the total amount of phosphorus in the alcohol-ether insoluble fraction of the virus estimated by the colorimetric tests, was about 30% greater than the amount of phosphorus found to be present by the direct estimation of total phosphorus.

Application of the orcinol reaction of Tillmans and Phillipi (16), standardized against glucose, to the trichloroacetic acid extract mentioned above indicated a carbohydrate content of about 7% of the weight of the virus. Estimations of carbohydrate by this method were not done on the trichloroacetic acid extracted residue.

#### *Chemical Fractionation of Radioactive Influenza Virus*

In order to determine the distribution of  $P^{32}$  between the phosphorus containing constituents of the labelled virus, two samples of purified radioactive virus were prepared from two groups of eggs which for Preparation I received 56,700 c.p.m. of  $P^{32}$  in each egg, and for Preparation II, 65,000 c.p.m. in each egg. Following the adsorption and elution technique with red cells previously described, the virus was subjected to two cycles of differential centrifugation, the virus being resuspended each time in 0.85% (w./v.) sodium chloride solution.

A known volume of Preparation I was then dried *in vacuo* from the frozen state, while an aliquot of Preparation II was agglutinated by dialysis against distilled water before drying in the same manner. Both dried preparations were then fractionated by the same methods as described for the nonradioactive virus. Total phosphorus and  $P^{32}$  estimations were carried out on each fraction; the specific activities were calculated and are shown in Table IV along with the results for total phosphorus. The total phosphorus figures for whole virus represent the total amounts of phosphorus in the aliquots taken for drying and for dialysis in Preparations I and II respectively. Phosphorus and radioactivity estimations on the alcohol-ether insoluble fractions were

TABLE IV

SPECIFIC ACTIVITIES OF FRACTIONS OF INFLUENZA VIRUS LABELLED WITH P<sup>32</sup>

Fraction	Preparation I		Preparation II	
	Total phosphorus, μg.m.	Specific activity, c.p.m./μg.m. P	Total phosphorus, μg.m.	Specific activity, c.p.m./μg.m. P
Whole virus	94.5	6.6	113.6	8.8
Petroleum ether soluble	46.0	3.0	17.9	3.6
Petroleum ether insoluble	1.5	Trace	33.5	3.6
Alcohol-ether insoluble	46.5	10.8	44.7	16.5

carried out on solutions of the material in 1.0 N potassium hydroxide, blank corrections being made for the very small natural radioactivity of potassium.

For Preparation II, the sum of the phosphorus figures for the three fractions is only 85% of the phosphorus in the whole virus. This difference could be accounted for by loss of virus in the manipulations attendant upon dialysis. Further, in this preparation the extraction of phospholipid from the alcohol-ether soluble fraction with petroleum ether seems to have been incomplete, judged by the amount of phosphorus containing material remaining in the petroleum ether insoluble fraction. The two experiments, however, are consistent in that they indicate the specific activity of the nucleic acid portion of the virus to have been 3.5–4.5 times that of the phospholipid.

In Preparation I, an attempt was made to purify the phospholipid by precipitation with magnesium chloride and acetone according to the method of Bloor (2) to find whether the specific activity would be changed by this treatment. After purification there was insufficient material left to allow an accurate radioactivity estimation. An attempt was also made to separate the PNA and DNA by the method of Schmidt and Thannhauser as described in a previous section. By far the largest proportion of the radioactivity was associated with the PNA fraction; it was impossible to get an accurate estimate of the small amount of activity remaining with the DNA.

## Discussion

As far as the chemical analysis of purified influenza virus has been carried in the present work the results are in agreement with previous work, except for the relative amounts of pentose and desoxypentose nucleic acids. On the assumption that the phosphorus of the virus was contained only in phospholipid and nucleic acid, the total amount of nucleic acid was found to be about 5%, calculated from the phosphorus content of the lipid free fraction. It has been demonstrated by Knight (7) that both types of nucleic acid are present since a nucleic acid fraction separated from over 2 gm. of purified virus gave positive color reactions for both pentose and desoxypentose.

Knight concluded from his analyses that the virus contained about 2.3% pentose nucleic acid, later revised to 3.0% (8), based on the use of the orcinol color test for pentose. While no figures were given, Knight mentioned that there appeared to be 9 to 10 times more pentose than desoxypentose present. The initial work on the virus by Taylor (15) indicated the presence of 2.1% desoxypentose nucleic acid. Thus it would appear that there is not yet complete agreement on the analysis of the nucleic acid portion of the virus.

In order to separate the two nucleic acids of the radioactive virus to determine their isotope contents and also to estimate the two nucleic acids by an independent method, the technique of Schmidt and Thannhauser has been applied to the lipid free fraction of the virus. From the analyses it appeared that the virus contained about 4.5% pentose nucleic acid and 0.3% desoxypentose nucleic acid. There was little or no phosphoprotein present. The same result was obtained with four different preparations of purified virus indicating, at least, that the technique of separation gave consistent results. Whether or not there was a clean separation of the two nucleic acids in the virus by this method cannot be deduced from phosphorus analyses alone. It has been demonstrated in the course of the present work that yeast and calf thymus nucleic acids can be separated almost quantitatively by the Schmidt and Thannhauser method, and similar results have been obtained with sheep liver pentose nucleic and calf thymus nucleic acids by McCarter and Steljes (9). However, it is not certain that the nucleic acids of influenza virus behave in the same manner as other nucleic acids when submitted to the action of dilute alkali.

In carrying out color tests on the separated nucleic acids of the virus in an attempt to confirm the above findings, it was observed that both the orcinol reaction for pentose and the diphenylamine reaction for desoxypentose might be subject to interference from other constituents of the virus. While the results of the colorimetric tests do not, therefore, give unqualified support for the estimations of the nucleic acids from phosphorus analyses of the separated acids, they are in agreement with the phosphorus analyses to the extent that they indicate the presence of much more pentose than desoxypentose nucleic acid.

The observation that in influenza virus containing  $P^{32}$  both phospholipid and nucleic acid fractions were labelled furnishes evidence, in addition to that already presented (5), that the isotope was incorporated into the structure of the virus. The nucleic acid fraction had a specific activity about four times that of the phospholipid, practically all the  $P^{32}$  of the nucleic acids appearing in the pentose nucleic acid. At the beginning of the work it was hoped that labelled influenza virus might be of assistance in determining whether the nucleic acids of the virus were synthesized after infection of the cell or incorporated into the virus directly from the constituents of the cell. However, the low specific activities of the radioactive virus preparations so far obtained, in addition to the relatively short half life of  $P^{32}$ , make analytical

work difficult. A method must be found for greatly increasing the specific activity of the virus before the problem will be amenable to this method of attack.

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# THE EFFECTS OF HIGH FAT DIETS AND COLD ENVIRONMENT ON THE ASCORBIC ACID CONTENT OF THE BROWN ADIPOSE TISSUE<sup>1</sup>

By EDOUARD PAGÉ AND LOUIS-MARIE BABINEAU

## Abstract

At room temperature, the ascorbic acid content of the brown adipose tissue is doubled when rats are fed a high fat diet. It is nevertheless lower than in tissues studied by others under somewhat similar conditions. In cold adapted rats, the brown body is considerably hypertrophied and the ascorbic acid content is from four to eight times higher than at room temperature. Under our experimental conditions, rats exposed to cold doubled the weight of their perirenal fat with little or no change in total body weight. It is concluded that cold stimulates fat metabolism and that both ascorbic acid and the brown adipose tissue are involved in the process.

## Introduction

We have already shown that prolonged exposure of rats to cold causes a considerable increase in the weight of the interscapular brown fat. This hypertrophy was associated with a rise in water and in nonfat dry matter content, and it was suggested that brown adipose tissue has some physiological function different from that of ordinary depot fat and that its activity is greatly enhanced in cold adapted animals (7). According to Wells (12), "Hoepke and Nikolaus (6) have recently demonstrated the probable presence of vitamin C in the hibernating gland of the hedgehog". Dugal and Thérien (3) have established the fact that the ascorbic acid concentration of several tissues increases in animals adapted to cold and it seemed opportune in the course of further experiments to measure the concentration of this vitamin in brown adipose tissue.

## Experimental

Four groups of 30 male albino rats each were used. Two groups received a low fat and a high fat ration, respectively, at room temperature while the two other groups received the same rations in the cold. The composition of the low fat ration was as follows: casein (untreated), 15.0; sucrose, 74.0; mineral salts, 4.0; CellufLOUR, 2.0; Mazola oil, 2.5; wheat germ oil, 2.5 gm. One hundred grams of ration contained: thiamine hydrochloride, 0.4; riboflavin, 0.5; pyridoxine hydrochloride, 0.5; nicotinic acid, 3.0; calcium pantothenate, 3.0; inositol, 10.0; 2-methyl-1, 4-naphthoquinone, 0.1; and choline chloride, 150 mgm. The high fat ration contained 40% fat as follows: Crisco, 25; Mazola oil, 10; and wheat germ oil, 5 gm. The level of other constituents was so adjusted at the expense of sucrose as to be similar to that of the low fat ration *on a calory basis*. A vitamin A and D supplement was given weekly by mouth.

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Average initial weight was 282 gm. for all groups. Two groups were put in the cold room at a temperature of about 8° C. ( $\pm 2^\circ$  C.) for the first 16 days. They were then exposed to temperatures varying between 0° and +3° C. until the end of the experiment when they were killed by decapitation between the 117th and 127th day. Only six rats could be sacrificed each day because of the many tissues taken for analysis. However, an equal number was taken from each group every day. Rats kept at room temperature were similarly sacrificed, some before and the others immediately after the cold adapted animals. Twenty additional rats were sacrificed at the beginning of the experiment and the fresh weights of various tissues were determined. Ascorbic acid was determined in the brown fat from some of the rats at room temperature and from others in the cold. Others were preserved for different analyses. The ascorbic acid was determined according to the method of Bessey and King (1). The perirenal fat (left side) was dissected and weighed in order to estimate the degree of fatness of the animals.

### Results

Changes in body weight are shown in Table I. These figures do not take into account the animals which died in the cold room and a few, at room temperature, which were discarded at an early date because of apparent ill

TABLE I

EFFECT OF DIET AND ENVIRONMENTAL TEMPERATURE ON BODY WEIGHT CHANGES IN THE RAT  
(Average initial body weight: 282 gm. for all groups)

	Number of rats	Final body weight (gm.)	Changes in body weight (gm.)	Significance of differences between groups (Value of "t")
<i>A—Room temperature</i>				
Low fat group	25	378	+ 96 $\pm$ 4.95*	
High fat group	26	411	+129 $\pm$ 9.38	3.11
<i>B—Cold room</i>				
Low fat group	24	277	- 5 $\pm$ 5.05	
High fat group	24	295	+ 13 $\pm$ 4.16	2.66

$$* \text{Standard error: } \sqrt{\frac{\sum d^2}{n(n-1)}}$$

health. It should also be made clear that although the animals in the cold room made little or no overall gain, they were actually recovering at the end some of the weight lost initially. They can therefore be considered as acclimatized.

Larger gains in body weight were made by the rats kept on a high fat diet. This confirms for rats exposed to cold the previous findings of Dugal, Leblond, and Thérien (2). At room temperature, where the difference is marked, the excess gain in body weight appears to be largely due to increased fat storage, if the weight of the perirenal fat is any indication of total fat depots (Table II).

TABLE II

## EFFECT OF DIET AND ENVIRONMENTAL TEMPERATURE ON PERIRENAL FAT (LEFT SIDE)

	Number of rats	Perirenal fat (gm.)	Value of "t"**	Perirenal fat per 100 gm. B.W.	Value of "t"
<i>A—Room temperature</i>					
Initial control group*	20	0.78 ± 0.10		0.27 ± 0.03	
Low fat group	25	4.57 ± 0.34	4.96	1.19 ± 0.07	
High fat group	26	7.49 ± 0.48		1.79 ± 0.09	4.98
<i>B—Cold room</i>					
Low fat group	24	1.58 ± 0.14		0.57 ± 0.05	
High fat group	24	1.89 ± 0.14	1.57	0.64 ± 0.05	1.00

\* This group was representative of the others and was sacrificed on the day the experiment was initiated.

\*\* Values of "t" exceeding 6.60 are found in all cases when comparing the low or high fat group in the cold with its control group at room temperature.

TABLE III

## EFFECT OF DIET AND ENVIRONMENTAL TEMPERATURE ON THE BROWN ADIPOSE TISSUE

	Number of rats	Brown fat (gm.)	Value of "t"**	Brown fat per 100 gm. B.W.	Value of "t"
<i>A—Room temperature</i>					
Initial control group	20	0.26 ± 0.015		0.09 ± 0.026	
Low fat group	25	0.80 ± 0.03		0.21 ± 0.01	
High fat group	26	0.91 ± 0.06	1.64	0.22 ± 0.01	0.06
<i>B—Cold room</i>					
Low fat group	24	1.30 ± 0.05		0.47 ± 0.02	
High fat group	24	1.52 ± 0.10	1.96	0.51 ± 0.03	0.90

\* Values of "t" exceeding 5.20 are found in all cases when comparing the low or high fat group in the cold with its control group at room temperature.

This effect of high fat rations has been demonstrated by many authors, notably Reed, Yamaguchi, Anderson, and Mendel (10), Pickens *et al.* (8), Williams *et al.* (13), and we had ourselves noted it previously (7). In the cold, the amount of perirenal fat is much less than at room temperature, nor is the difference between diets of any statistical significance. Surprisingly enough, the perirenal fat (left side) amounts to 0.6% of the total body weight in both groups exposed to cold as compared to 0.3% in the control rats killed initially (Table II), although average body weights are nearly the same in all cases. While it has been known for a long time that the water content of the body decreases in the cold, it is interesting to find in the present instance what appears to be a corresponding replacement of water loss by gain in fat.

It is seen in Table III that the weight of the interscapular brown adipose tissue increases with age and body weight at room temperature, with no significant differences between groups. In the cold, the increase is so marked that the brown fat weighs over 60% more, in absolute values, than in the larger control rats kept at room temperature and over twice as much on a body weight basis, irrespective of the ration.

Table IV shows the ascorbic acid content of brown adipose tissue in seven rats from each group at room temperature and in six rats from each group in

TABLE IV

## EFFECT OF DIET AND ENVIRONMENTAL TEMPERATURE ON THE ASCORBIC ACID CONTENT OF THE BROWN ADIPOSE TISSUE

	Room temperature	Cold room	Increase in the cold (%)	Value of "t"
<i>A—Ascorbic acid concentration (γ/gm.)</i>				
Low fat group	65.3 ± 14.8*	305.6 ± 41.1	368	5.50
High fat group	119.4 ± 19.5	353.7 ± 36.9	196	5.62
Increase on high fat ration (%)	83	16		
Value of "t" (low vs. high fat)	2.22	0.89		
<i>B—Ascorbic acid content (γ)</i>				
Low fat group	52.4 ± 6.4	437.5 ± 64.1	735	5.98
High fat group	103.2 ± 11.4	455.2 ± 62.7	341	5.52
Increase on high fat diet (%)	97	4		
Value of "t" (low vs. high fat)	3.88	0.19		

\* Standard error:  $\sqrt{\frac{\sum d^2}{n(n - 2)}}$

the cold. These rats were taken at random and it so happened that in the cold, the average weight on the low fat diet was higher than the average weight for the whole groups (1.43 gm. as against 1.30), while it was lower for the high fat group (1.28 gm. as against 1.52 for all animals). It is nevertheless abundantly clear that prolonged exposure to cold increases considerably the ascorbic acid content of the brown adipose tissue. It is further noted that at room temperature, the ascorbic acid content is approximately doubled on the high fat ration.

### Discussion

Variations in the weight of the perirenal fat with diet and temperature will be discussed in a separate paper dealing with the quantitative relationship between this tissue and other fat deposits.

It is sufficient to note here: (a) that the weight of the perirenal fat is much less in the cold than at room temperature although in the former case, the brown adipose tissue is considerably hypertrophied; (b) that following exposure to cold the animals increased their fat stores without gaining weight. Thus, initial loss in body weight in the cold may represent to a large measure a loss of water which is gradually compensated by increased fat deposition.

From the increase in weight of the brown adipose tissue in the cold and from the much higher concentration in ascorbic acid, it can safely be concluded that cold stimulates the activity of this tissue. If we compare our values for ascorbic acid with those reported by Dugal and Thérien (3) for other rat tissues under somewhat similar conditions, we find that at room temperature the concentration in brown fat is much less than in the testes, kidneys, or liver. In the cold, it becomes equal to that of the liver and is exceeded only by that of the adrenals. It seems, therefore, that insofar as functions connected with ascorbic acid are concerned, *the brown adipose tissue is relatively unimportant at room temperature but displays in the cold an activity level comparable to that of the liver.*

Dugal and Thérien (3) have already shown the close relationship existing between adaptation to cold, adrenal function, and ascorbic acid. On the other hand, Fawcett and Jones (5) have demonstrated by cytological studies that the "maintenance of the normal complement of lipid in brown adipose tissue depends upon the functional integrity of the adrenal cortex". It is, therefore, more than probable that the *stimulating effect of cold on this tissue is mediated through the adrenals.*

There is also good reason to believe that the brown adipose is involved in fat metabolism. In hibernating animals brown fat attains its maximum size just prior to hibernation and at a time when vast stores of fat are being accumulated. It degenerates during winter sleeps (9). This "hibernating gland" may therefore be concerned with *preparation* for, rather than *maintenance* of hibernation. Fawcett and his associates (4, 11) have brought forward excellent evidence that it may be a preferred site of fat synthesis: thus, in hyperglycemic but nondiabetic rats, as well as in normal or diabetic

animals after the administration of insulin, the deposition of glycogen prior to its conversion into fat is far greater in brown adipose tissue than in ordinary depot fat.

Finally, Dugal and Thérien (3) have shown that at room temperature, a high fat ration always leads to a higher concentration of ascorbic acid in tissues, as compared to a low fat ration, this difference tending to disappear in the cold where concentrations are much higher. Our own results fully confirm this finding with the additional information that increase in ascorbic acid concentration in the cold is greater in the brown adipose tissue than in any other organs studied. Because of the obvious relationship between this tissue and fat metabolism, we feel justified in considering that the particularly marked rise in its ascorbic acid content in the cold indicates that *this vitamin is linked with some phase of fat metabolism.*

### Summary and Conclusion

In rats adapted to a cold environment, there occurs a considerable hypertrophy of the brown adipose tissue and an even higher rise in its ascorbic acid content. Under our experimental conditions, these rats increased their fat stores with little or no change in body weight. At room temperature, the ascorbic acid content of the brown adipose tissue is doubled on a high fat ration. It is suggested, on the basis of these findings and from other considerations discussed above, that this tissue is involved in fat metabolism, that exposure to cold stimulates its activity and finally, that ascorbic acid is intimately linked with some phase of fat metabolism.

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## FURTHER WORK ON THE NUTRITION OF DUCKLINGS

A.—LIPOTROPIC FACTORS. B.—SULPHUR AMINO ACID REQUIREMENTS<sup>1</sup>

By J. M. DEMERS AND R. BERNARD

### Abstract

Using a purified diet deficient in choline and containing 18% casein, it has been found that in the duckling: (a) betaine and aminoethanol have little or no lipotropic activity, are poor growth factors, and cannot prevent perosis; (b) mono-methylaminoethanol and dimethylaminoethanol, on the other hand, have well marked lipotropic activity. Both substances stimulate growth and are anti-perotic, dimethylaminoethanol being the most active of the two in these respects; (c) ducklings, like chicks, appear unable to methylate aminoethanol to any extent; (d) a level of 28% casein is more satisfactory than one of 18% in the prevention of fatty infiltration of the liver in ducklings.

Betaine and methionine have a definite lipotropic activity, when fed to ducklings along with a semipurified diet containing peanut meal as the only natural ingredient. The substitution of yellow corn meal to sucrose in the above semipurified diet inhibits the lipotropic activity of methionine, without affecting that of betaine.

The availability of a protein low in sulphur amino acids has resulted in the preparation of a more hypolipotropic diet. On this diet, ducklings presented livers containing, on the average, 24% of lipids. Methionine added to this diet is strongly lipotropic, but is without effect on growth. Methionine will promote growth only in presence of choline.

Ducklings like chicks and poult appear to be able to convert methionine to cystine. In presence of 0.4% cystine, approximately 0.5% methionine is required for normal growth.

### Introduction

Using a purified diet containing 18% casein, Bernard and Demers (2) have shown that choline has a definite lipotropic action in ducklings. Under these conditions, betaine presented a slight lipotropic activity, but the results were not significant. On the other hand, methionine, when added to a purified diet containing 9% casein or to semipurified diet containing 15% casein and yellow corn meal, increased the total lipids and the severity of fatty infiltration of the livers. The purpose of this paper is to present the results of additional studies on lipotropism in ducklings and preliminary information on the sulphur amino acids requirements of this avian species.

### Materials and Methods

The experimental procedures followed are similar to those already described in detail (2). These procedures deal with the preparation of the diets, care of the animals, the determination of liver lipids, and the preparation of microscopic sections of the liver.

An arbitrary fatty infiltration index (F.I. index) has been resorted to: microscopic examination of frozen sections of liver sections stained for fat

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permits the grading of fatty changes according to scale proposed by Drill and McCormick (4). An average score is calculated for each group and is designated as fatty infiltration index. This index varies from 0.0 to 4.0 and represents a mean fatty infiltration for each group of animals.

The composition of the basal diets used is given in Table I. Diets R-49, R-66, and R-73 resemble each other in being deficient in choline, though to a different degree. Diet R-73 is particularly hypolipotropic. Besides being

TABLE I  
COMPOSITION OF BASAL DIETS

Ingredients	Basal diet number			
	R-49	R-66	R-73	R-76
	Composition			
%				
Main ingredients				
Sucrose	47	50	51	51
Casein, Labco	18	10	28	28
Isolated soybean protein*		15		
Peanut meal**	4	4		
Liver fraction L***				
Fish oil blend†	2	2	2	2
Salts IV††	5	5	5	5
CaH <sub>4</sub> (PO <sub>4</sub> ) <sub>2</sub> H <sub>2</sub> O	1	1	1	1
Ruffex	3	3	3	3
Vitamins	Mgm. per 100 gm. of diet			
Thiamine hydrochloride	0.4	0.4	0.4	0.4
Riboflavin	0.8	0.8	0.8	0.8
Calcium pantothenate	2.5	2.5	2.5	2.5
Nicotinic acid	4.0	4.0	4.0	4.0
Pyridoxine hydrochloride	0.4	0.4	0.4	0.4
α-Aminobenzoic acid	2.0	2.0	2.0	2.0
Biotin	0.02	0.02	0.02	0.02
Inositol	100.0	100.0	100.0	100.0
2-Methyl naphthoquinone	0.1	0.1	0.1	0.1
α-Tocopherol	10.0	10.0	10.0	10.0
Pteroylglutamic acid			0.1	0.1
Choline chloride				300.0

\*"Alpha protein". The Glidden Company, Chicago, Ill.

\*\* Expeller process, 45% protein. Planters Edible Oil Co., Suffolk, Va.

\*\*\* A source of the unknown vitamins of the B-complex. Wilson Laboratories, Chicago, Ill.

† Vadol Type No. IV, 3000 A, 400 D (A.O.A.C. chick units). Blended fish oils fortified with vitamin D<sub>3</sub>. Ayerst, McKenna and Harrison, Montreal, Que.

†† Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B. J. Biol. Chem. 138 : 459. 1941.

choline-free, it contains a protein which is low in cystine and methionine. The purified diet R-49 contains about 0.004% choline (calculated), i.e., that brought by the Liver Fraction L. The simplified (semipurified) diet R-66 has a choline content (calculated) about 10 times that of the preceding diet. This is accounted for by the peanut meal and the Liver Fraction L. Finally, diet R-76 includes all the ingredients of diet R-73 plus 0.3% added choline chloride and is well suited for the study of cystine and methionine requirements.

## Results and Discussion

### A. Lipotropic Factors

(a). The results of the first three series of experiments are presented in Fig. 1 and Table II. Each experiment includes groups of animals started on the

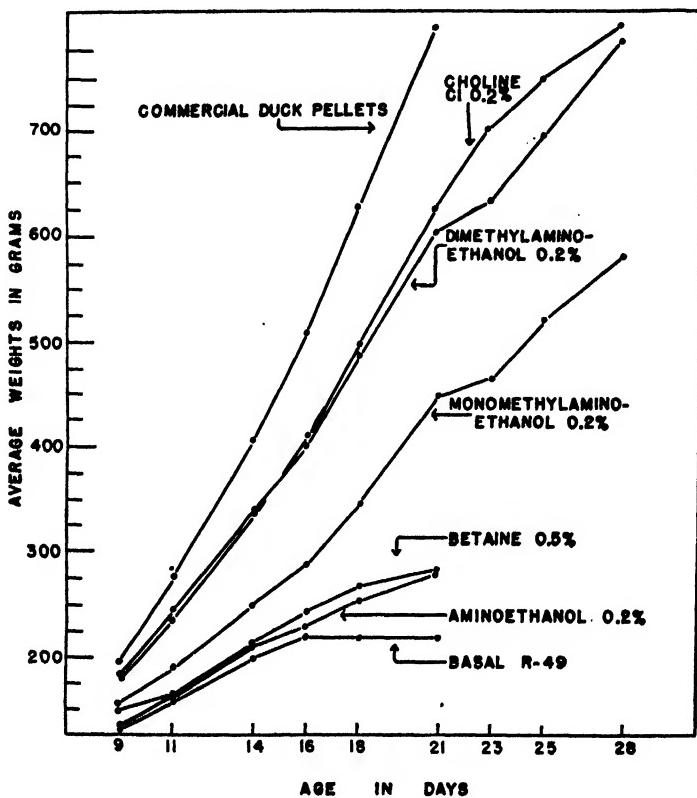


FIG. 1. Growth curves of ducklings fed different lipotropic factors.

same day. Using purified diet R-49, the lipotropic effect of betaine, aminoethanol, monomethylaminoethanol, and dimethylaminoethanol were studied, as well as the effect of raising the casein level. Observations were also made on growth and on incidence of perosis.

TABLE II

EFFECT OF SOME LIPOTROPIC FACTORS WHEN ADDED TO AN 18% CASEIN, PURIFIED DIET

Expt.*	Supplement to basal diet R-49	F.I. index**	Liver lipids, %***	††	Birds with perosis Birds on experiment	Average weight, gm.	
						5 days	21 days
I	None	3.2	14.5 ± 1.91††	0.40	10/10	102	222 ± 12
	Betaine 0.5%	2.5	12.6 ± 2.40		9/10	105	284 ± 18
	Commercial duck pellets only	0.0	6.8 ± 0.13		0/6	83	800 ± 24
II	Aminoethanol 0.2%	3.1	12.5 ± 1.85	0.73	6/8	104	280 ± 16
	Choline chloride 0.3%	1.4	7.4 ± 0.76		0/8	86	574 ± 21
	Choline chloride 0.3%						
III	Casein, Labco, 10%	0.6	6.7 ± 1.34	3.33	0/8	85	601 ± 30
	Choline chloride 0.2%	1.4	8.2 ± 0.43		0/8	89	625 ± 22
	Monomethylaminoethanol 0.2%	2.1	8.2 ± 0.97		5/8	95	447 ± 24
	Dimethylaminoethanol 0.2%	1.6	8.5 ± 0.33		2/8	95	604 ± 35

\* Birds of Experiment III were killed at 28 days, others at 21 days.

\*\* Fatty infiltration index of the liver.

\*\*\* Per cent wet weight.

† t &gt; 2.30 significant by comparison with control group.

†† Standard error  $\sqrt{\frac{\sum d^2}{n(n - 1)}}$ .

1. Betaine. Previous work (2) on ducklings indicated that betaine has a slight lipotropic activity though the results were not statistically significant. The investigation was repeated, and it was found that this quaternary amine has little or no lipotropic activity and has no antiperotic properties. A small but significant stimulation of growth was noticed.

2. Aminoethanol, monomethylaminoethanol, and dimethylaminoethanol.

In Table II, the percentages of these amines refer to the free bases. However, they were neutralized with concentrated hydrochloric acid prior to their addition to the basal diet.

It is well known that there exist striking differences between the rat and the chick in respect of the utilization of these precursors of choline.

In 1941, Stetten (14) found in the rat, that aminoethanol can accept labile methyl groups for the synthesis of choline. In the chick, on the contrary, Jukes (5) observed that this primary amine cannot serve as a precursor of choline, when added to a purified diet enriched in methionine.

Using a semipurified diet containing yellow corn meal and peanut meal as natural ingredients, McGinnis, Norris, and Heuser (11) noticed in chicks that aminoethanol does not improve growth and has very slight antiperotic activity. On the other hand, Kummerow, Weaver, and Hornstead (10) recently reported that the same amine is antiperotic and stimulates growth, when fed along with a natural diet rich in fats.

Jukes and coworkers (6, 7) have demonstrated with chicks that dimethylaminoethanol is as efficient as choline in the prevention of perosis, but slightly inferior to it as a growth factor. Monomethylaminoethanol is antiperotic, but promotes growth only in the presence of methionine. These last authors conclude that the chick is unable to methylate aminoethanol, but can accomplish the second step in the synthesis of choline, that is, the transformation of monomethylaminoethanol into dimethylaminoethanol.

From the standpoint of comparative physiology, it was thus of interest to investigate the effect of feeding these amines to ducklings, particularly with respect to their influence on liver lipids.

The present studies with ducklings indicate that aminoethanol (Table II, Expt. II) has little or no lipotropic activity, that it is a poor growth factor and that it does not prevent perosis. It appears that ducklings are similar to chicks in being unable to methylate aminoethanol. Monomethylaminoethanol and dimethylaminoethanol (Expt. III) exhibit a lipotropic activity equal to that of choline. Dimethylaminoethanol is as good as choline in promoting growth, and only slightly inferior to the latter in antiperotic properties. On the other hand, 60% of the birds treated with monomethylaminoethanol showed symptoms of perosis and the average growth for the group was only two-thirds that of the control group fed choline chloride.

Working with chicks, Jukes *et al.* (7) found that monomethylaminoethanol prevents perosis without any improvement on growth. It appears that chicks and ducklings react differently when fed a diet supplemented with monomethylaminoethanol.

### 3. Effect of increasing the casein content of the diet on fatty infiltration of the liver.

In a preceding study (2), the authors have reported that the supplementation with 0.3% choline chloride of diet R-49 containing 18% casein did not bring the percentage of total lipids entirely back to normal, and that fatty infiltration was still persistent. Consequently, an attempt was made to assess the lipotropic value of casein by increasing the percentage of this protein by 10%. It will be seen (Expt. II) that the group of ducklings fed the 28% casein diet presented an average of 6.7% for liver lipids. This is as low as that of the group fed the commercial diet. Moreover, the fatty infiltration of the liver nearly disappeared. Growth was good and nearly equal in both groups.

It is thus concluded, that the purified diet containing 18% casein and supplemented with 0.3% choline chloride is still deficient in one or more lipotropic factors.

#### (b) Lipotropic activity of betaine and methionine in presence of semipurified diets.

The following experiments were suggested by the observations of McGinnis, Norris, and Heuser (12) in chicks. These authors reported that betaine or methionine do not improve in any way the nutritive value of a purified diet. On the other hand, when the same substances were added to a semipurified diet containing peanut meal or yellow corn meal, they improved growth markedly and were effective in preventing perosis.

A similar study was undertaken in ducklings making use of semipurified diet R-66 which contained 15% peanut meal as the natural ingredient. The protein content of this basal diet is approximately 16%.

Table III shows that the addition of 0.5% betaine (Expt. IV) reduces the liver lipids to 9.2% as compared with 16.8% for the unsupplemented group.

TABLE III

## EFFECT OF PEANUT MEAL AND YELLOW CORNMEAL ON THE LIPOTROPIC ACTIVITY OF BETAINES AND METHIONINE

Expt.	Group No.	Supplement to basal diet R-66	F.I. index	Liver lipids, %	<i>t</i>	Birds with perosis Birds on experiment	Average weights, gm.	
							5 days	28 days
IV	1	None	3.5	16.8 ± 1.06	6.39	8/8	105	770 ± 39
	2	Betaine 0.5%	1.9	9.2 ± 0.54		6/8	95	679 ± 42
	3	DL-methionine 0.5%	1.5	8.7 ± 0.49		6/8	96	801 ± 48
V	4	Corn meal 50%	0.9	8.4 ± 0.93	1.42	5/8	96	986 ± 46
	5	Corn meal 50%				6/8	95	820 ± 29
		Betaine 0.5%	0.3	7.0 ± 0.32		6/8		
	6	Corn meal 50%				8/8	85	816 ± 41
		DL-methionine 0.5%	2.0	12.3 ± 1.75				

*Comparison of liver lipids: Groups 1 and 4, t = 5.94.*

" 2 and 5, t = 3.45.

" 3 and 6, t = 3.33.

Methionine is as effective as betaine, if not more, in this respect. However, both groups of birds still showed evidence of slight fatty infiltration. These results constitute the first evidence of the lipotropic activity of betaine and methionine in ducklings.

Substitution of yellow corn meal to the sucrose of basal diet R-66 (Expt. V) lowered the liver lipids from 16 to 8%. This lipotropic effect of the yellow corn meal is probably due to its choline content. It was surprising to find that the simultaneous addition of yellow corn meal and methionine favors the production of fatty livers. This new information confirms the antilipotropic effect of methionine in presence of yellow corn meal as noticed in a previous work (2). It is suggested that the zein content of the corn meal may cause an imbalance in amino acid makeup of the diet which is reflected by the appearance of fatty livers. Zein is known to contain large quantities of leucine and

glutamic acid. Furthermore, Beveridge *et al.* (3) have showed that the lipotropic effect of a diet is determined not only by its sulphur-containing amino acids but also by its adequacy in other respects, particularly the sulphur-free amino acids in the protein.

Since the majority of the birds in Experiments IV and V were perotics, it is suggested that if any choline was synthesized from betaine or methionine, it must have been used up for some other purpose, probably the lipotropic function. Finally, Table III shows that all groups grew well, especially the fourth one which presented the highest average weight at 28 days of age.

In summary, the experiments with semipurified diets have revealed that betaine and methionine are lipotropic in presence of peanut meal. One may surmise that this natural ingredient contains a suitable methyl acceptor or that it corrects an amino acid imbalance. The replacement of sucrose by yellow corn meal in diet R-66 did not alter the lipotropic activity of betaine but inhibits that of methionine and even renders it antilipotropic.

(c) Study of the lipotropic activity of cystine and methionine using a diet containing isolated soybean protein.

In a last series of experiments on fatty infiltration of the liver of ducklings a more hypolipotropic diet (R-73) was made use of. The Liver Fraction L of previous diets was dispensed with and the casein replaced with a soybean protein known to be deficient in cystine and methionine (9). Table IV shows an average of 24% for the liver lipids of the unsupplemented group. This

TABLE IV

EFFECT OF CYSTINE AND METHIONINE WHEN ADDED TO A CHOLINE-FREE DIET, CONTAINING 28% ISOLATED SOYBEAN PROTEIN

Supplement to basal diet R-73	F.I. index	Liver lipids, %	<i>t</i>	Birds with perosis Birds on experiment	Average weights, gm.	
					5 days	21 days
None	3.6	24.2 ± 3.98		5/8	107	193 ± 6
L-cystine 0.5%	2.9	15.3 ± 2.75	1.84	8/8	105	162 ± 5
DL-methionine 0.5%	0.8	6.6 ± 0.42	4.39	8/8	88	209 ± 9

value exceeds that of the similar groups of preceding experiments by 8 or 11% respectively and confirms the marked hypolipotropic nature of basal diet R-73. It should be recalled, however, that a 9% casein diet (2) produced livers containing on the average, 34% lipids for the basal group.

The group of ducklings fed the cystine supplement had a liver lipid value of 15%, though the results are not significant when compared to the control group. This result is surprising, for cystine is recognized in the rat (8, 13) as antilipotropic, especially when added to diets low in protein and rich in fats.

Methionine at the level of 0.5% was very effective as a lipotropic factor. The liver lipids of this group being 6.6% with a low fatty infiltration index of 0.8%. This last experiment further shows that the lipotropic effect is related to other constituents of the diet particularly the quality and quantity of the protein used.

Table IV further reveals that on a choline-free diet, methionine does not improve growth, while cystine has a definite retarding effect. Finally, most ducklings of the three groups were perotic.

This last series of experiments with basal diet R-73 brings additional evidence for the lipotropic activity of methionine in ducklings. It is surprising to note that the action of methionine is limited to the prevention of fatty livers to the exclusion of growth. If the lipotropic effect of this amino acid is exerted through the biosynthesis of choline, it is possible that this latter substance is used up entirely for the removal of fat from the livers and that none is left for growth and prevention of perosis. It would be of interest to know which of these functions has priority over the others.

#### B. The Sulphur Amino Acid Requirements of Ducklings

Using a basal diet (R-76) containing 28% isolated soybean protein and a sufficient amount of choline, it was possible to investigate the requirements of ducklings for cystine and methionine. The basal diet resembles that used by Kratzer *et al.* (9) in their study with turkey poult. According to these authors, it contains 24% crude protein, 0.36% methionine, and 0.05% cystine, and growth is limited by methionine and cystine deficiencies.

The results are presented in Table V and Fig. 2. The basal diet alone or supplemented with 0.5% cystine are inadequate for growth. Excellent growth

TABLE V  
SULPHUR AMINO ACID REQUIREMENTS OF DUCKLINGS

Supplement to basal diet R-76	F.I. index	Liver lipids, %	Birds with perosis Birds on experiment	Average weights, gm.		<i>t</i>
				4 days	22 days	
None	1.0	7.1 ± 0.32	2/5	103	253 ± 11	
DL-methionine 0.1%	1.1	7.4 ± 0.32	1/7	102	464 ± 25	7.38
DL-methionine 0.3%	0.7	6.9 ± 0.14	0/6	94	541 ± 39	7.08
DL-methionine 0.5%	0.1	7.0 ± 0.05	3/7	85	402 ± 31	4.45
L-cystine 0.5%	0.1	6.4 ± 0.80	0/8	94	296 ± 25	1.64
L-cystine 0.5%						
DL-methionine 0.1%	0.3	7.0 ± 0.39	1/7	82	536 ± 30	8.73

was obtained with the basal diet enriched with 0.1 or 0.3% methionine, the last level being more satisfactory. It thus appears that like chicks, turkeys, and other animals, ducklings are able to convert methionine into cystine and

that cystine may not substitute for methionine. It should be emphasized, however, that methionine improves growth only in the presence of an adequate supply of choline. It is surprising to note that a level of 0.3% methionine is

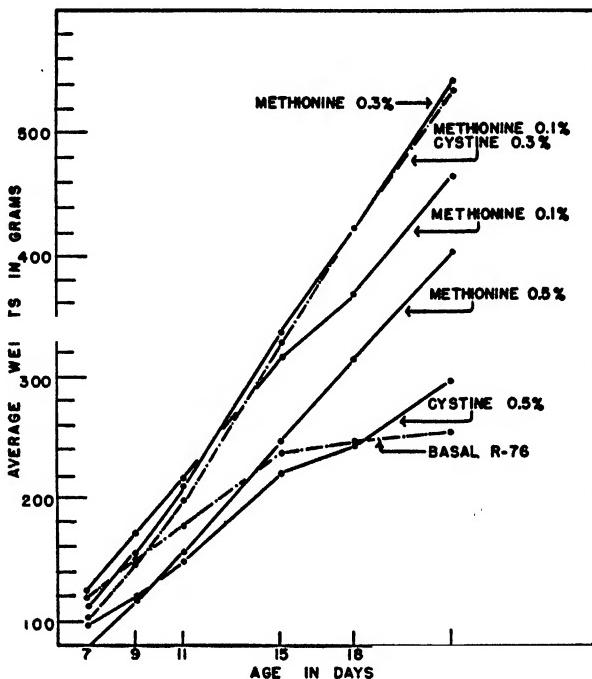


FIG. 2. *Effect of different levels of methionine and cystine on growth of ducklings.*

more satisfactory for growth than that of 0.5%. An excess of methionine may create an amino acid imbalance which is translated by inhibition of growth and higher incidence of perosis as observed in the fourth group of birds. Finally, addition of 0.3% cystine and 0.1% methionine to the basal diet gives excellent growth equal to that obtained with 0.3% methionine only. Thus, the simultaneous addition of the two sulphur amino acids results in a significant economy in methionine.

Examination of the liver fatty infiltration indices suggest that methionine and cystine add to the lipotropic effect of choline alone except for the group which received 0.1% methionine. However, this is not corroborated by the liver lipid values which are close to normal in all groups.

Taking into account the sulphur amino acid contents of the basal diet, it is suggested that in the relative absence of cystine, the methionine requirement of ducklings are of the order of 0.8% cystine. In the presence of 0.4% cystine, a level of 0.5% methionine is apparently adequate for normal growth. These values are close to those found by Kratzer *et al.* (9) for turkey poult and Almquist (1) for chicks.

### Acknowledgments

The authors wish to thank Ayerst, McKenna, and Harrison for a supply of Blended Fish Oils; Merck and Co., for biotin and  $\alpha$ -tocopherol; Hoffmann-La Roche for biotin,  $\alpha$ -tocopherol, riboflavin, pantothenic acid, thiamine hydrochloride, and pyridoxine hydrochloride; Lederle Laboratories for pteroyl-glutamic acid (Folvite); Carbide and Carbon Chemicals Limited for mono-methylaminoethanol.

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AN ANTIBIOTIC PRODUCED BY *MICROCOCCUS EPIDERMIDIS*<sup>1</sup>

By L. J. LOEB, A. MOYER, AND R. G. E. MURRAY

**Abstract**

A stable antibiotic was produced by a strain of *Micrococcus epidermidis* that showed a wide range of activity against Gram-positive organisms. A mucoid *Streptococcus pyogenes* was used as test organism. This strain could be made resistant by being grown in increasing concentrations of antibiotic but the organism reverted to its original susceptibility immediately on transfer to medium without antibiotic. There was no antiluminescent activity when tested on *Photobacterium fischeri*. The test organism was not lysed by the antibiotic. The active substance was dialyzable, was remarkably heat stable, and was soluble only in water or, providing water was present, in solvents that were completely miscible with water. Purification was successful only to the extent of removing a number of inactive fractions by differential solubilities. The activity was destroyed by trypsin but not by pepsin. The physical and chemical data make it probable that the substance is a polypeptide of low molecular weight.

Two strains of *Micrococcus epidermidis* have been described (4) that produced a stable antibiotic. The activity was restricted to a range of Gram-positive organisms and was diffusible through cellophane. The results of further study of the antibiotic produced by one of these strains (2921) is presented in this paper.

**Methods**

The media used and the general methods of production and titration have been described by Murray and Loeb (4). The antibiotic was produced by a cellophane sac technique in Difco nutrient broth supplemented by equal amounts of Difco proteose peptones No. 2 and No. 3 added to 1%. By this method a cell-free broth was obtained. A mucoid *Streptococcus pyogenes* (M. Strep. 9) was used as the test organism in plate tests on blood agar and in the dilution assays in dextrose broth.

**Observations****PRODUCTION**

Assay during production showed that the titer rose to a maximum and remained at that level. After 14 days' incubation at 37° C. the broth usually attained a titer greater than 1/200 and was harvested as "crude antibiotic". This material was stored in screw-top vessels ready for subsequent treatment. No drop in activity has been noted during storage. In fact, aliquots stored at room temperature and at 4° C. have maintained a constant titer for more than 18 months.

Proteose-peptone seemed to provide some factor necessary for satisfactory production of the antibiotic. Production in a nutrient broth base was compared

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Contribution from the Department of Bacteriology and Immunology, University of Western Ontario, London, Ont. Aided by a grant from the National Research Council of Canada.

to the same medium with the addition of 0.5% and 1.0% of proteose-peptone (Fig. 1). These supplements allowed a more rapid production and an increased titer.

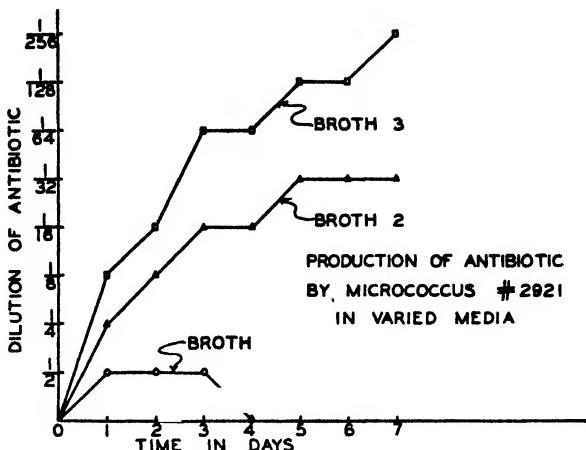


FIG. 1. A graph showing titers of antibiotic produced by *Micrococcus epidermidis* (2921) in varied media.

Broth 1: Nutrient broth.

Broth 2: Nutrient broth with 0.5% proteose-peptone.

Broth 3: Nutrient broth with 1.0% proteose-peptone.

### BIOLOGICAL ACTIVITY

The antibiotic was active against a number of Gram-positive bacteria, as shown by the streak plate tests (4). *Corynebacterium diphtheriae* (five strains) and *Mycobacterium tuberculosis* (H37RA and B.C.G.) were not affected nor were any of the Gram-negative organisms that were tested. It was found also that a bactericidal action on the test *Streptococcus* was exhibited when concentrations of antibiotic more than twice the minimum inhibitory level was used. There was no evidence of lysis and the cells did not show any change in staining character using Gram's method, even when suspended in a bactericidal concentration of antibiotic for four days. The test organism became more resistant on repeated passage through media containing gradually increasing concentrations of the crude antibiotic, but after one subculture to media without antibiotic the organism was again fully sensitive. Antibiotic 2921 does not have any antiluminescent activity against *Photobacterium fischeri*, a property (3) that is peculiar to some antibacterial agents.

### PHYSICAL AND CHEMICAL PROPERTIES

#### Stability

The stability of the crude antibiotic at room and refrigerator temperature has already been noted. It was unaffected by autoclaving for 20 min. at 121° C. or by boiling for 30 min. at pH 2, pH 7, and pH 12.

## CRUDE ANTIBIOTIC (10 ML.)

Evaporate to a moist residue (1 ml.) and take up with 9 ml. of pyridine; centrifuge.

Supernatant - add equal amount of ether; centrifuge.

Residue - no activity.

Supernatant - no activity.

Residue - dry, moisten and take up with 10 ml. 95% ethanol; centrifuge.

Supernatant - add equal amount of ether; centrifuge.

Residue - no activity.

Supernatant - no activity.

Residue - dry and take up with 10 ml. distilled water. Yield, 50% of activity.

FIG. 2. Flow sheet diagram of partial purification of the crude antibiotic from *Micrococcus epidermidis*.

### Solubility

The activity was readily soluble in water at all pH reactions but could not be extracted with any of the common organic solvents from the dried residue of the crude antibiotic. However, the inhibitory substance was soluble in those solvents which are completely miscible with water if the dried residue was moistened slightly with water beforehand. These solvents included methyl, ethyl, and propyl alcohols, acetone, pyridine, and dioxane. It was not soluble in solvents that are only partially miscible or not miscible with water: ethyl ether, chloroform, butyl alcohol, ethyl acetate, carbon tetrachloride, or cyclohexane. The fractions were tested by filter paper disc method against *M. Strep. 9* on blood agar plates.

### Extraction and Purification

Considerable difficulty was experienced in attempting purification because of the solubility characteristics of the substance. The antibiotic was dialyzable through cellophane into both normal saline and distilled water, but only about 20% of the activity diffused in 24 hr. Phosphotungstic acid caused a heavy precipitate that included the antibiotic activity, but this residue proved to be

difficult to handle. Attempts to purify the antibiotic by partition chromatography were unsuccessful because no suitable solvent was found. The activity was adsorbed on "Norit" (activated animal charcoal) and was eluted by pyridine at pH 7, but not by water, 50% ethyl acetate or water-saturated butanol at pH 2, pH 7, or pH 10. The yield of antibiotic was low (10% of the activity) after elution with pyridine.

Using a moistened dried residue of the crude antibiotic, it was found that pyridine and ethyl alcohol precipitated impurities leaving the inhibitory substance in solution. The addition of ether caused a yellowish precipitate containing the antibiotic which could be redissolved in water from this residue. A process of partial purification seemed most feasible, giving approximately 50% yield of the inhibitory substance. A flow sheet diagram of this process is presented in Fig. 2. A brownish viscous fluid was obtained when the partially purified material was concentrated to 1/20th of the original volume.

#### *Effect of Proteolytic Enzymes and Serum*

The crude antibiotic was digested with 0.1% trypsin (Difco) at pH 8 and 1% pepsin (Difco 1 : 10,000) at pH 2, and plain broth was treated similarly as controls. The antibiotic was inactivated after one hour of incubation with trypsin at 37° C., while the aliquot digested with pepsin retained its activity. Incubation with sheep serum (1 : 1) for 18 hr. reduced the activity of the mixture by 50%.

#### Discussion

Antagonistic micrococci have been described for over 60 years. The early reports gave little indication of the nature of the substances, and strains were described in terms of antibacterial spectrum and possible applications. Inhibitory strains of *Micrococcus pyogenes* have been most frequently reported although Dujardin-Beaumetz (2) described a nonpathogenic *Micrococcus* that inhibited most Gram-positive organisms and many Gram-negative strains. Recently Su (5) described a nonpathogenic *Micrococcus* producing a dialyzable antibiotic that was heat stable and soluble in alcohol and chloroform. The activity of the purified fractions was not affected by horse serum, trypsin, or pepsin, and the action was bacteriostatic, affecting many Gram-positive organisms. The antibiotic produced by *Micrococcus epidermidis* described here does not correspond in antibacterial spectrum, physical or chemical properties to those described by Dujardin-Beaumetz and by Su.

The solubility of the antibiotic places it in a group of substances described by Waksman (6) which are "soluble in water at different reactions, and insoluble in ether. These substances usually represent polypeptides, proteins, organic bases, or adsorption compounds on protein molecules. Most of them have not been isolated in pure state".

The differential action of pepsin and trypsin may provide further information as to its chemical constitution. Bergmann (1) explained that trypsin can act on peptide links formed from the carboxyl group of either arginine

or lysine, but requires that the second amino group of the dibasic amino acid unit be free. Pepsin does not act on this linkage. This evidence suggests that the active substance may be a low molecular weight polypeptide that is heat stable and dialyzable through cellophane.

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## OXYGEN UPTAKE OF RAT MAMMARY TISSUE SLICES<sup>1</sup>

BY JULES TUBA, HERBERT E. RAWLINSON, AND LORNA GLEN SHAW

### Abstract

An *in vitro* study has been made of the oxygen uptake of mammary gland tissue of female rats in various experimental states. Because of the very high proportion of fat in mammary tissue the values of  $Q_o$ , are determined on a fat-free as well as a water-free basis, thus providing a more accurate measure of the oxygen consumption of this tissue. The oxygen utilization by mammary gland of pregnant animals is increased approximately three times over the activity in the normal, or resting, gland. This increase is maintained during lactation and a return toward normal levels occurs during postlactational involution. The response to *p*-phenylenediamine indicated that during lactation the increased energy requirements decreased the reserves of the cytochrome system in mammary tissue. There is a well developed mammary gland in adult male rats; but the average fat content and response to *p*-phenylenediamine of the tissue are almost identical with values for adult female rats. The use of *p*-phenylenediamine as a histological stain for the cytochrome system in mammary tissue is described.

### Introduction

There has been comparatively little investigation of the respiratory activity of rat mammary gland tissue *in vitro*, probably because of the difficulties encountered in preparing the tissue for such studies. Kleiber, Smith, and Levy (5) measured the oxygen consumption of rat mammary tissue during pregnancy and lactation. They found on a dry-weight basis that mammary tissue from lactating rats manifested greater values of  $Q_o$ , than tissue obtained from the pregnant animals. Attempts to correlate oxygen uptake with nitrogen content of the glands were unsuccessful because of the variable milk protein content. Folley and French (4) carried out a survey of the respiratory metabolism of slices of rat mammary tissue during pregnancy, lactation, and postlactational involution. Their values for  $Q_o$ , also based on dry weight, were greater than those reported by Kleiber *et al.*

Investigations in this laboratory have indicated that rat mammary tissue contains a very large proportion of fat which may be varied by such factors as pregnancy, lactation, and weaning. It was further noted by use of a histological technique that very little of the activity due to the cytochrome system is associated with the fatty material of the glands, and that almost all the oxygen uptake associated with this system is concentrated in the epithelial cells. In view of these findings, we decided to investigate the  $Q_o$ , levels of rat mammary tissue on a water-free, fat-free basis. The endogenous respiration of this tissue was generally low. Excess *p*-phenylenediamine was added to the reaction vessels in order to saturate the cytochrome system. It was considered that, by comparing the endogenous oxygen uptake with the

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Contribution from the Department of Biochemistry and the Department of Anatomy, University of Alberta, Edmonton, Alta. The investigation was supported by a grant from the National Cancer Institute of Canada.

oxygen consumption following the addition of *p*-phenylenediamine, a more accurate measurement could be obtained of the cytochrome reserves in rat mammary tissue.

## Experimental

### I. MANOMETRIC METHODS

Male and female adult albino rats of the Wistar strain were used. The animals were killed by decapitation and the required tissues were removed at once and placed in ice-cold calcium-free Ringer's solution (pH 7.4). Inguinal glands were always used, since they can be completely separated from muscle tissue.

The preparation of mammary tissue for measurement of oxygen uptake was a difficult problem. The use of a homogenate was attempted and abandoned because the large amount of fatty material present permitted some of the brei to creep up the walls of the center well of the reaction vessel until it eventually came in contact with concentrated base. We found, as have other workers, that slicing techniques are extremely difficult to apply to mammary glands. It was decided to use scissors to cut several small pieces from approximately the same portion of the gland in each case. Although the tissue was not in pieces as thin as those advocated by Folley and French, the technique did permit rapid transfer from the animals to the reaction vessel, and we found replicates to be in good agreement. Moreover, *p*-phenylenediamine was added in all determinations, and it is known to penetrate readily the fatty material of the gland.

The pieces of tissue were transferred to Warburg reaction flasks, each of which contained 1.8 ml. calcium-free Ringer-phosphate-glucose solution. The center well of each flask contained 0.2 ml. 20% potassium hydroxide and a piece of fluted filter paper. The use of *p*-phenylenediamine as a substrate for the cytochrome system necessitated the use of a control flask, identical with the respiring flask except for the omission of tissue and potassium hydroxide, in order to allow correction for autoxidation. The side arms of control and experimental flasks contained 0.3 ml. of a freshly prepared solution of *p*-phenylenediamine which had been adjusted to pH 7.4 with 0.1 *N* hydrochloric acid. The final concentration of *p*-phenylenediamine in the reaction flasks was 0.2% or  $1.85 \times 10^{-2} M$ , since this amount of the substrate has been found sufficient to saturate the cytochrome system in mammary tissue under all experimental conditions studied by us. The vessels were gassed for 10 min. with 100% oxygen, in conformity with the work of Folley and French, placed in a water bath at 37° C., and shaken for 15 min. to bring their contents to equilibrium. The manometers were then read for the next 30 min., by which time a steady rate of endogenous oxygen utilization was measured. At this point *p*-phenylenediamine was tipped in from the side arm of each flask. The first five minute reading following the addition was disregarded and then the manometers were read at five minute

intervals for 20 min. usually, during which time the rate of oxygen consumption was linear.  $Q_{O_2}$  and  $Q_P^{O_2}$  values for mammary tissue were calculated on the basis of constant oxygen consumption for three consecutive five minute readings. Estimation of the activity of each gland was always done in duplicate.

At the conclusion of all experiments, the pieces of tissue from each vessel were transferred to a Gooch crucible, which had a watch glass set underneath it. The watch glass served to retain any fatty material which separated from the tissue during the overnight drying period. After the material had been dried in an oven at 110° C., it was weighed. It is possible that there may be a slight error in this weight in the case of lactating glands due to the milk error mentioned by Folley and French. However, the tissue was in small pieces, and we believe that most of the milk was leached out in the reaction vessel during the experiment. After the dry weight was determined the tissue was heated with ether in a beaker to remove the bulk of the fat. Finally the remaining fat was removed in a Soxhlet apparatus and the weight of the dried, fat-free tissue was then determined. The values of  $Q_{O_2}$  in  $\mu\text{l./mgm./hr.}$  were calculated on the basis of (a) dry weight (as usually reported in the literature); (b) dry, fat-free weight; and, (c) dry, fat-free weight following the addition to the reaction vessel of *p*-phenylenediamine.

## Results

### Female Rat Mammary Tissue

Oxygen consumption was measured in the mammary tissue obtained from adult female rats in various experimental conditions. The values of  $Q_{O_2}$  determined on the three bases listed above are presented in Table I.

TABLE I

OXYGEN CONSUMPTION ( $Q_{O_2}$ ) AND RESPONSE TO *p*-PHENYLENEDIAMINE ( $Q_P^{O_2}$ ) OF MAMMARY TISSUE OF ADULT FEMALE RATS IN VARIOUS EXPERIMENTAL STATES

(Numerical values are averaged and in Columns (b), (c), and (d) the standard error of the mean is indicated)

Experimental state	Number of rats	(a) $Q_{O_2}$ on dry weight basis	Fat as % dry weight	(b) $Q_{O_2}$ on fat-free dry basis	(c) $Q_P^{O_2}$ on fat-free dry basis	(d) Increase of (c) over (b)*
Normal	11	0.9	87	3.7 ± 0.3	9.6 ± 0.78	5.9 ± 0.8
Pregnant (10-14 days)	4	1.8	84	10.2 ± 0.1	17.7 ± 1.28	7.5 ± 1.3
Lactating (18-21 days)	6	5.2	47	10.1 ± 0.26	13.0 ± 0.42	2.9 ± 0.4
Seven days after weaning	2	0.9	83	5.1 ± 0.12	14.8 ± 0.72	
Castrated	4	0.2	95	3.9 ± 0.43	11.5 ± 0.56	

\* The results were analyzed statistically by the method for the significance of the difference of means of small samples. (R. A. Fisher, *Statistical methods for research workers*. 9th ed. Oliver and Boyd, Ltd., Edinburgh and London. 1944) It was found that the increases in column (d) were highly significant. The increases in  $Q_{O_2}$  (b) and  $Q_P^{O_2}$  (c) for pregnant and lactating animals, as compared with the corresponding normal values, were also found to be significant.

Kleiber *et al.* found a threefold increase, on a dry-weight basis of  $Q_{O_2}$  of mammary tissue of lactating rats compared with the pregnant animals, and our results are in agreement with theirs. An eightfold increase is reported by Folley and French. Table I indicates that if the  $Q_{O_2}$  values are estimated on a fat-free basis (Column b) there is a threefold increase over normal in pregnancy, and this is maintained at a constant level during the latter part of lactation, at least. There is a return towards normal values seven days after weaning, which, in this laboratory, occurs 21 days after the birth of the young.

The mammary glands of castrated female rats contain more than the normal amount of fat and the tissue has as a result an abnormally low dry-weight  $Q_{O_2}$ .

The very marked enhancement of oxygen uptake which followed the addition of *p*-phenylenediamine indicates, according to Craig, Basset, and Salter (3) that there are in normal, or resting, mammary tissue considerable reserves of the cytochrome system. As indicated in the last column of Table I there is a small but significant rise of these reserves during mid-pregnancy followed by a pronounced lowering during lactation when there are special demands for energy in the glands.

#### *Male Rat Mammary Tissue*

Since it was noted histologically that the male rat has a fairly well developed mammary gland, determinations were run on the male mammary tissue. For five adult male rats we found that the average fat content (90%) and  $Q_{O_2}^0$  ( $10.8 \pm 0.55$ ) were practically identical with corresponding values for adult female mammary tissue.

### II. HISTOLOGICAL METHODS

During the oxygen consumption studies reported above, it was noticed that when *p*-phenylenediamine was added to the reaction vessels the mammary gland slices appeared to darken only in certain well defined areas. It seemed probable that the color development was associated with oxidation of *p*-phenylenediamine by the cytochrome system and, therefore, that the actual site of oxidation might be determined histologically.

Mammary tissue was obtained from normal adult female rats in the usual way. Small pieces were placed in a test tube and shaken for an hour with 1.8 ml. Ringer-phosphate-glucose solution and 0.3 ml. *p*-phenylenediamine solution, prepared as above for manometric experiments. The tissue was left in the reaction mixture overnight, and in the morning it was washed with distilled water and then treated with 0.1 N hydrochloric acid for at least 15 min. to leach out the unoxidized *p*-phenylenediamine. The stained pieces of mammary gland were then blotted dry and cleared overnight in oil of origanum. They were further cleared in xylol and mounted in piccolyte.

As a control, pieces of the same gland were treated with potassium cyanide in order to inhibit oxidation through the cytochrome system. The tissue

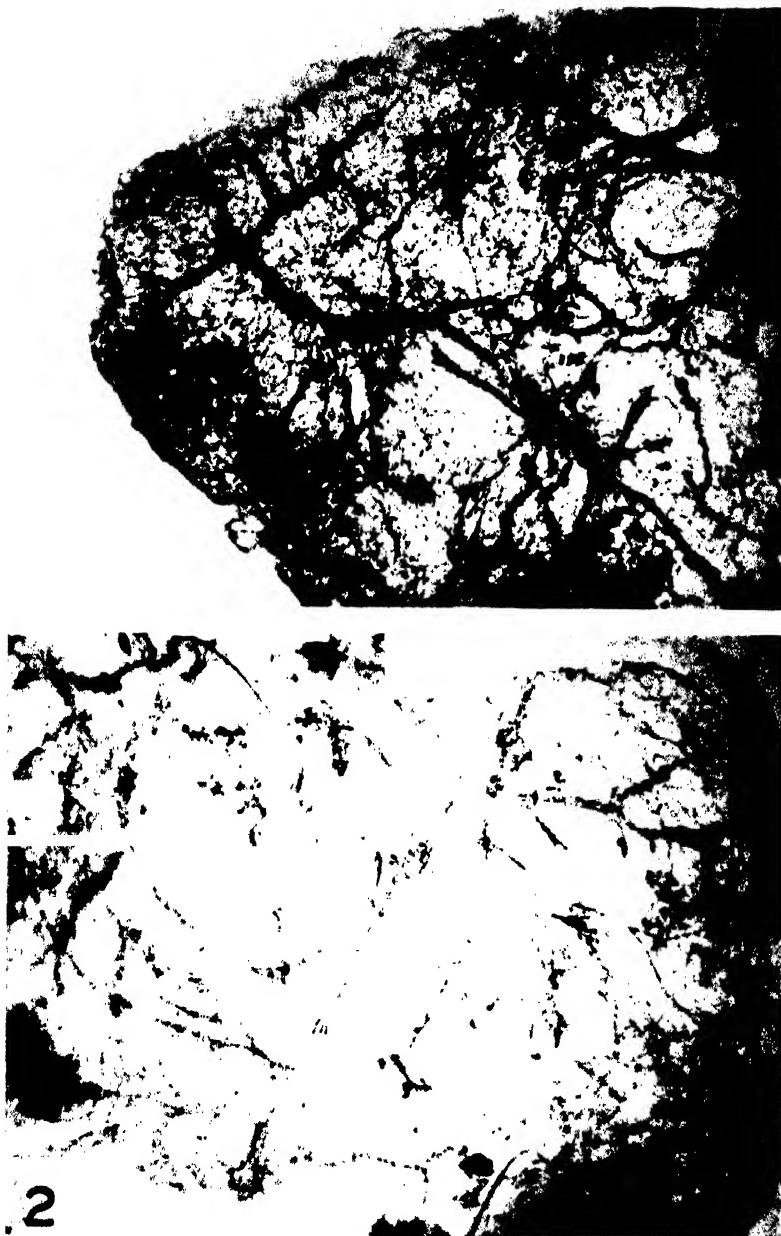


FIG. 1. Section of inguinal mammary gland of adult female rat, stained with *p*-phenylenediamine,  $\times 25$ . The epithelial gland tree is outlined by concentration of the oxidized *p*-phenylenediamine.

FIG. 2. Section of gland used for Fig. 1,  $\times 25$ . Treatment as with the tissue in Fig. 1 did not show up gland tree because the potassium cyanide prevented oxidation of *p*-phenylenediamine, but as the figure shows, subsequent staining by hematoxylin revealed that mammary epithelium was present.



was immersed in 1.5 ml. Ringer-phosphate-glucose solution and 0.3 ml. 0.3 M potassium cyanide for 30 min. before the addition of 0.3 ml. *p*-phenylenediamine. The pieces of gland were then treated exactly as the tissue in the active tube.

### Results

Figs. 1 and 2 are photomicrographs of the *p*-phenylenediamine stained and the cyanide treated tissues respectively.

The staining seen in Fig. 1 is due to the oxidation of *p*-phenylenediamine and it is clear that the reaction is concentrated in the epithelial cells of mammary gland, occurring only to an insignificant degree in the fat. Some pieces of tissue were treated with potassium cyanide as outlined above and when these were examined histologically no precipitate was found in the epithelial cells. To make sure that mammary gland tissue was present these pieces were subsequently stained in whole mounts by hematoxylin and the presence of a gland tree was established (Fig. 2). This shows that the catalytic mechanism involved in the oxidative response to *p*-phenylenediamine is cyanide sensitive. Various workers, including Commoner (2), consider that cyanide inhibition of tissue oxidation is associated with the cytochrome system. The histological results would justify the assumption that the staining by *p*-phenylenediamine is mediated through the iron-containing cyanide-sensitive cytochrome system in mammary gland epithelium.

Others have reported on stains for the cytochrome system. Montagna and Noback (6, 7), used the nadi reagent ( $\alpha$ -naphthol and dimethyl-*p*-phenylenediamine) to stain cytochrome oxidase in mast cells. Becker (1) also used the nadi reaction but stated that it did not allow any conclusion as to the localization of the cytochrome system.

Because of the diluting effect of fat, which we have shown by staining to contain practically no cytochrome, it is felt that values of  $Q_{O_2}$ , as reported in this paper, give a precise indication of the respiratory activity of mammary tissue.

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**THE USE OF  
PRISCOLINE (2-BENZYLIMIDAZOLINE HYDROCHLORIDE)  
AS A TEST IN OCCLUSIVE ARTERIAL DISEASE<sup>1</sup>**

By J. DOUPE AND R. M. CHERNIACK

**Abstract**

In 19 subjects with chronic occlusive arterial disease an intramuscular injection of 75 mgm. Priscoline (2-benzyl-2-imidazoline) was found in most cases to produce a greater rise in toe temperature than did the body warming procedure of Gibbon and Landis. In five subjects the vasodilating property of Priscoline was found to be superior to that of spinal anesthesia. In four cases the results with Priscoline predicted the effect of sympathectomy with greater accuracy than did the body warming test. The superiority of the Priscoline test was ascribed to the presence of denervation sensitivity due to a disturbance of sympathetic innervation occurring in association with occlusive arterial disease.

**Introduction**

Smithwick (16) has recently drawn attention to a group of patients with occlusive peripheral arterial disease who respond poorly to vasodilatation tests but nevertheless benefit remarkably from sympathectomy. It is obvious, that, for such cases, some new diagnostic test is desirable. The simplest and most reliable tests in modern-day use are the reflex thermal test and spinal anesthesia. A new test should be superior to either of these.

Currently, a number of new drugs are under investigation as to their effect on the peripheral circulation. One of these, Etamon (tetraethylammonium chloride), gave early promise (1, 4) of being a useful adjunct to diagnosis but later reports (2, 6) were disappointing. More recently Priscoline (2-benzyl-imidazoline hydrochloride) which is adrenolytic and sympatholytic (10, 11) has been used as a vasodilating agent in the treatment of peripheral vascular disease (9, 13) and its diagnostic usefulness has also been suggested (8). The present study was therefore undertaken to assess its diagnostic value by comparing its action to the effects of the reflex thermal test, spinal anesthesia, and sympathectomy.

**Subjects and Methods**

The subjects were the 19 patients shown in Table I, all of whom were referred with a diagnosis of organic occlusive arterial disease of the lower extremities but were otherwise unselected except that they had to be available for two or more tests. Note was made of the presence or absence in the feet of a disturbance of the appreciation of light touch and pin prick. Those cases in which the examiner had difficulty in estimating sensory loss were classified as questionable.

Skin temperature was used as the index of blood flow and was measured by means of copper constantan (gauge 34) thermocouples held in contact with the pads of exposed digits by narrow strips of adhesive tape on the dorsum of the second phalanx. Temperatures were recorded every two to four minutes

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Contribution from the Department of Medicine and the Department of Physiology and Medical Research of The University of Manitoba and from the Winnipeg General Hospital, Winnipeg, Canada.

from two digits of each foot and from the right index finger, and read to  $0.1^{\circ}\text{C}$ . Rectal temperature was recorded by a thermocouple and read to  $0.01^{\circ}\text{C}$ . Room temperature was kept constant at  $19^{\circ} - 21^{\circ}\text{C}$ . and measured by a thermocouple suspended several feet above the parts under observation.

Subjects reclined in the constant temperature room clad only in a hospital gown. To provide a base line, recordings were made for 10 to 30 min. before a test. The reflex thermal test of Gibbon and Landis (7) was performed by immersing the left arm in water at  $44^{\circ}\text{C}$ ., and by covering the trunk and proximal parts of the extremities with blankets. When, after prolonged heating, a definite plateau of maximum skin temperature was attained in the toes and the control finger, the blankets were removed and the left arm placed in cold water at  $18^{\circ}\text{C}$ . This stimulated reflex vasoconstriction, thereby testing the integrity of the sympathetic nerves. On another day, each subject received 75 mgm. Priscoline intramuscularly, and the effect was observed for one and one-half to two hours. Blood pressure, recorded at five minute intervals, was little affected. Five of the subjects received 100 mgm. Novocaine intrathecally. In all, analgesia was produced at least to the level of the 10th thoracic dermatome and the response was observed for one hour or longer. The effect of lumbar sympathectomy on skin temperature was observed in four of the subjects two to four weeks after operation at which time the reflex thermal test was repeated.

### Results

During each procedure note was taken of the height to which the skin temperature rose. A comparison of the maximum temperatures attained during the reflex thermal test and the Priscoline test is shown in Fig. 1 and Table I.

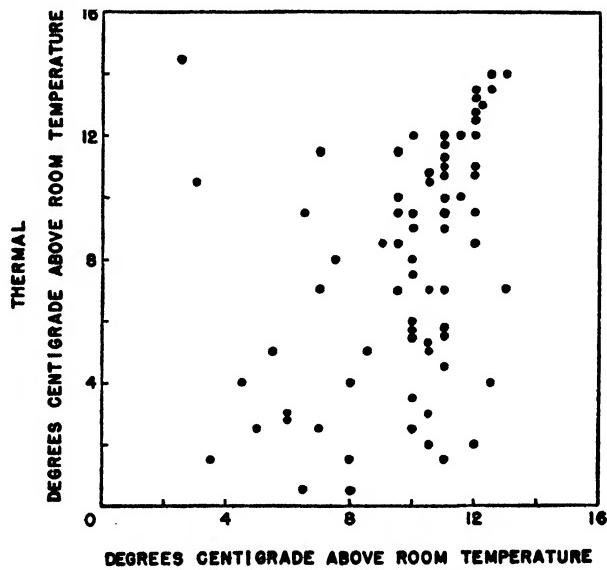


FIG. 1. A comparison of the maximum temperatures attained with the reflex thermal test and the intramuscular injection of 75 mgm. of Priscoline.

TABLE I  
SUBJECTS AND RESULTS

Case No.	Diagnosis	Age	Sex	Clinical summary	Digit*	Sensation**	Vasomotor responses**	Thermal	Prisoline	Maximum temperature, °C.	Difference
1	Arteriosclerosis	58	M	Pain, numbness left leg for 2 years. Pulsations: L-, R+	LT4 LT1 RT4 RT1	N N N N	N N N N	31.5 30.5 34.5 34.5	24.0 27.5 33.0 33.0	- 7.5 - 3.0 - 1.5 - 1.5	
2	Arteriosclerosis	51	M	Pain in both calves and ankles for 4 years. Pulsations: L+, R+	LT4 LT1 RT4 RT1	N N N N	N N N N	31.0 31.5 32.0 30.5	32.0 32.0 31.0 31.0	+ 1.0 + 0.5	
3	Arteriosclerosis	75	M	Pain in both calves, worse in left, for 1 year. Pulsations: L-, R-	LT4 LT1 RT4 RT1	N N N N	N N N N	26.0 25.5 32.0 32.0	31.5 32.0 33.0 33.0	+ 5.5 + 6.5 + 1.0 + 1.0	
4	Arteriosclerosis	61	M	Pain in both calves, worse in left, for 3 years. Pulsations: L-, R-	LT5 LT1 RT5 RT1	?	?	30.5 31.0 35.0 34.5	33.0 32.5 34.0 33.5	+ 2.5 + 1.5 - 1.0 - 1.0	
5	Arteriosclerosis	66	M	Pain in both calves, worse in left, for 4 years. Pulsations: L-, R-	LT4 LT1 RT4 RT1	?	?	24.0 23.5 33.5 33.0	30.5 31.0 33.0 32.5	+ 6.5 + 7.5 - 0.5 - 0.5	
6	Arteriosclerosis	69	M	Duodenal ulcer. Pain in right calf and cold feet for 2 years. Pulsations: L-, R-	LT4 LT1 RT4 RT1	A A A A	N N N N	33.5 34.0 33.0 30.0	33.0 33.0 32.0 32.0	- 0.5 - 1.0 - 1.0 + 2.0	
7	Arteriosclerosis	63	M	Pain and numbness left leg for 3 years. Burning left sole for 1 year. Pulsations: L-, R-	LT4 LT1 RT4 RT1	?	A A A ?	26.0 25.0 30.5 29.5	31.5 29.0 32.5 30.5	+ 5.5 + 4.0 + 2.0 + 1.0	

\* LT4 means left fourth toe, RT1, right first toe, etc.

\*\* N indicates normal, A abnormal, and ? questionable sensation or vasomotor response.

TABLE I—Continued  
SUBJECTS AND RESULTS—Continued

Case No.	Diagnosis	Age	Sex	Clinical summary	Digit*	Sensation**	Vasomotor responses**	Thermal	Priscoline	Maximum temperature, °C.	Difference
8	Arteriosclerosis	64	M	Pain in both calves, worse in left, for 4 years. Pulsations: L—, R—	L14 LT1 RT4 RT1	?	A	26.5 24.5 28.0 26.5	31.0 31.0 32.0 32.0	+ 4.5 + 6.5 + 4.0 + 5.5	
9	Arteriosclerosis	69	M	Pain in both calves for 3 years. Numbness and cold feet—5 months. Pulsations: L+, R+	L14 LT1 RT4 RT1	A	A	23.0 22.5 28.0 25.0	33.0 32.0 34.0 33.5	+ 10.0 + 9.5 + 6.0 + 8.5	
10	Buerger's	40	M	Amputated right leg 2 years. Numbness and pain in left leg for 9 months. Pulsations: L—	L14 LT1	N N	N N	31.5 29.5	31.5 33.0	0 + 3.5	
11	Buerger's	40	M	Pain in left great toe for 7 months. Pulsations: L—, R—	L14 LT1 RT4 RT1	N N N N	N N N N	25.5 26.0 32.5 35.5	25.0 26.5 28.0 23.5	- 0.5 + 0.5 - 4.5 - 12.0	
12	Buerger's	30	M	Pain in right thigh and calf on walking for 3 months. Slight numbness. Pulsations: L+, R—	L15 LT1 RT5 RT1	N N N N	N N N N	35.0 33.0 22.5 23.5	33.5 33.0 29.0 28.0	- 1.5 0 + 6.5 + 4.5	
13	Buerger's	47	M	Pain in left calf and right foot, numb cold feet for 1 year. Pulsations: L—, R—	L14 LT1 RT4 RT1	?	A	33.0 32.0 32.5 31.0	32.0 32.0 30.5 30.5	- 1.0 0 - 2.0 - 0.5	
14	Buerger's	50	M	Pain in both legs and right great toe; worse at night. Pulsations: L—, R—	L14 LT1 RT3 RT1	A A A A	A A A A	32.0 30.0 31.0 24.0	32.0 31.0 27.0 24.0	0 + 1.0 + 3.0 + 3.0	

\* LT4 means left fourth toe, RT1, right first toe, etc.

\*\* N indicates normal, A abnormal, and ? questionable sensation or vasomotor response.

TABLE I—*Concluded*  
SUBJECTS AND RESULTS—*Concluded*

Case No.	Diagnosis	Age	Sex	Clinical summary	Digit*	Sensation**	Vasomotor responses**	Maximum temperature, °C.		Difference
								Thermal	Priscoline	
15	Diabetes Arteriosclerosis	59	M	Pain in both calves, particularly right for 9 years. Cold and numbness, particularly right leg—3 years. Pulsations: L—, R—	LT4 LT1 RT4 RT1	A A A A	A A A A	28.0 29.5 28.0 33.0	30.5 30.5 31.5 31.0	+ 2.5 + 0.5 + 3.5 - 2.0
16	Diabetes Arteriosclerosis	63	F	Numbness and cold feet for 10 years. Ulcers both ankles for 9 months. Pulsations: L—, R—	LT4 LT1 RT4 RT1	A A A A	A .N A A	23.0 26.5 21.5 21.5	31.5 32.0 27.5 29.0	+ 8.5 + 5.5 + 6.0 + 7.5
17	Diabetes Arteriosclerosis	66	F	Pain, numbness, and cold feet for 3 years. Pulsations: L—, R—	LT4 LT1 RT4 RT1	N A A A	N A A A	31.0 26.0 29.5 23.5	30.5 29.5 26.0 24.5	- 0.5 + 3.5 + 2.5 + 2.0
18	Diabetes Arteriosclerosis	48	F	Amputated right leg, 4 years. Numbness left leg, 1 year. Gangrene left great toe. Pulsations: L—	LT4 LT1	A A	A A	29.0 28.0	28.5 28.0	- 0.5 0
19	Diabetes Arteriosclerosis	57	M	Burning feeling in both legs and feet for 2 years. Pulsations: L—, R—	LT4 LT1 RT4 RT1	?	A A A ?	28.5 27.0 27.0 29.0	31.0 31.0 31.0 31.0	+ 2.5 + 4.0 + 4.0 + 2.0

\* LT4 means left fourth toe, RT1, right first toe, etc.

\*\* N indicates normal, A abnormal, and ? questionable sensation or vasomotor response.

It will be seen that 44 of the 72 digits attained a higher temperature with Priscoline than with reflex heating. In 31 of these the difference was greater than 2° C. In only four digits did the effect of the reflex thermal test exceed that of the Priscoline by more than 2° C.

Fig. 2 shows that in the 20 digits tested the effect of Priscoline was greater than that of reflex heating or spinal anesthesia.

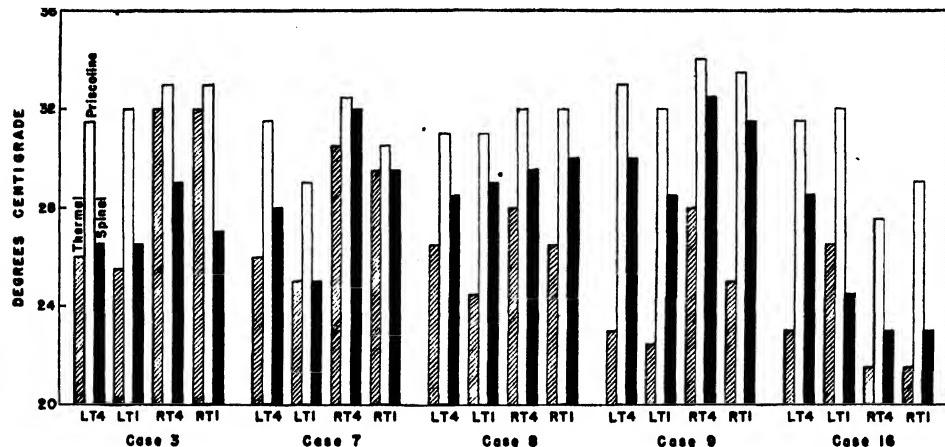


FIG. 2. A comparison of the maximum temperatures attained in toes with the reflex thermal test, intramuscular Priscoline, and spinal anesthesia.

The preoperative and postoperative results in the four subjects who were sympathectomized are shown in Table II. The average error of the reflex thermal test in the prediction of the operative result was 4.2° C. while with Priscoline it was 2.2° C.

TABLE II

THE EFFECT OF PREOPERATIVE TESTS AND SYMPATHECTOMY ON SKIN TEMPERATURE  
(TEMPERATURES GIVEN IN °C. ABOVE ROOM TEMPERATURE)

Case No.	Digit	Thermal	Priscoline	Sympathectomy
5	LT4	3.0	9.5	5.5
	LT1	2.5	10.0	8.0
10	LT4	10.5	10.5	12.0
	LT1	8.5	12.0	12.5
12	RT5	1.5	8.0	10.5
	RT1	2.5	7.0	9.0
18	LT4	8.0	7.5	10.0
	LT1	7.0	7.0	10.0

During the course of the experiments it was observed in certain cases that the vasomotor behavior of the digits was abnormal. This abnormality ordinarily consisted of a failure of the vessels of certain toes to constrict in response

to a general cold stimulus although the vessels of the control finger and other toes showed a good vasoconstrictor response. In a smaller number of cases, the vasomotor behavior was judged to be abnormal because there was an absolute absence of any vasodilatation in response to prolonged heating. That the failure to increase the blood flow was not due to arterial disorder in this group of cases was shown by the ability of other procedures such as Priscoline to produce vasodilatation. These abnormal vascular reactions resembled those reported by Doupe (5) and Richards (12) in individuals with peripheral nerve lesions. This and the abnormalities of sensation suggested the presence of a neuropathy.

It was therefore of interest to observe whether the efficacy of Priscoline could be related to any evidence of this postulated nerve lesion. Table III indicates

TABLE III

THE EFFECT OF DENERVATION ON THE RELATIVE EFFICACY OF THE INJECTION OF PRISCOLINE AND THE REFLEX THERMAL TEST

Evidence of denervation	Total number of digits	Number of digits		
		Priscoline less than reflex	Priscoline equal to reflex	Priscoline greater than reflex
No sensory loss	25	4	16	5
Questionable sensory loss	22	0	10	12
Definite sensory loss	25	0	11	14
Normal vasomotor behavior	40	4	25	11
Abnormal vasomotor behavior	32	0	12	20

that, in comparison to the cases with normal innervation, those with sensory loss or abnormal vasomotor behavior had a higher proportion of digits in which the response to Priscoline was greater than the response to the reflex thermal test. Results within 2° C. of each other were considered equal.

### Discussion

It is apparent from the above results that in cases of clinical peripheral occlusive arterial disease Priscoline had a greater vasodilating effect than either reflex heating or spinal anesthesia. In a limited number of cases it was further found, in agreement with Smithwick (16) that the reflex thermal test tended to underestimate the effect of sympathectomy. In contrast to this the results of the injection of Priscoline approximated those obtained by sympathectomy.

The explanation for the superiority of Priscoline in certain cases may depend on the existence of a state of hypersensitivity to adrenaline. In the first place it has been established by Cannon (3) that sympathetic denervation greatly increases the sensitivity of vessels to adrenaline and secondly there is evidence that a peripheral nerve lesion exists in many cases of occlusive arterial disease. Thus Shumacker (15) noted a defect in vasoconstrictor ability in cases of

arteriosclerosis and Buerger's disease, while Rundle (14) has reported a similar condition associated with minimal sensory changes in diabetics. In the present series similar evidence indicative of a peripheral nerve lesion was found. Therefore it is not surprising that Priscoline, being adrenolytic as well as sympatholytic was more potent than either sympathetic inhibition or block.

It appears paradoxical that sympathectomy should increase the blood flow to digits already denervated, though this has been shown to occur in cases of proven traumatic nerve lesions (5). The explanation for this may be that sympathectomy prevents the liberation of adrenaline from endings adjacent to those which are hypersensitive. In addition it is possible that the increased warmth of the whole extremity impedes the vasoconstricting action of a cold environment to which peripherally denervated vessels are also hypersensitive (5).

These considerations suggest that the resemblance between the effects of Priscoline and sympathectomy, though real and valuable, is nevertheless fortuitous. It appears that most vasodilatation tests are unsatisfactory because they are likely to induce an increased secretion of adrenaline and because their short duration does not permit the spasm due to the cold environment to be overcome. For these reasons tests which depend simply on blocking or inhibiting vasoconstrictor impulses do not necessarily give results corresponding to those obtained by sympathectomy. On the other hand, if part of the effect of Priscoline is attributable to its adrenolytic property, it too would not necessarily duplicate the effects of sympathectomy. Nevertheless the present results suggest that it does this more closely than do the other procedures.

The results reported here strongly support the contention of Green and Ogle (8) that Priscoline is useful in distinguishing between the functional and organic elements in occlusive arterial disease. This appears to be particularly true where the spasm is secondary to denervation sensitivity.

### Acknowledgments

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## THE HAEMOGLOBINS OF THE FOETUS AND NEWBORN<sup>1</sup>

By F. D. WHITE, G. E. DELORY, AND L. G. ISRAELS<sup>2</sup>

### Abstract

In addition to the two forms of haemoglobin previously recognized in the blood of the foetus and newborn, a third form has been detected by following the rate of reaction with sodium hydroxide. This component appeared in 33 of the 66 samples studied but could not be detected in the remainder. No explanation could be found for this anomaly. Confirmation was obtained of the presence of a second form of haemoglobin in adult blood. This was shown to differ from the most refractory fraction of foetal blood. It is suggested that the older terminology is no longer sufficiently definitive and an alternative scheme is proposed.

### Introduction

It has been known for many years that the haemoglobin of human blood can exist in at least two forms. One of these forms, "foetal" or "refractory" haemoglobin, occurs mainly in the blood of the foetus and newborn infant; while the other, "adult", "labile", or "later" haemoglobin, is present in the adult and in children over four months of age (10).

The two haemoglobins have been shown to differ in many ways, notably in their oxygen dissociation curves (2, 12, 7), the rate of spread of their monomolecular films (3), their antigenicity (5), their amino acid composition (14), and in their reaction with alkali (15). The corresponding methaemoglobins also differ in regard to solubility and crystalline structure (11). It is now generally accepted on the grounds of evidence derived from these procedures, together with the investigations of Haurowitz (8, 9), that the differences in these haemoglobins reside in the globin portion of the molecule—the ferroporphyrin group being identical.

Following the qualitative studies of Von Kruger, Haurowitz determined the relative concentrations of these haemoglobins in bloods containing both forms, by utilizing the difference in their rates of conversion into alkaline globin haematin. This principle was modified and extended by Brinkman and Jonxis (3), Baar and Lloyd (1), and recently by Ponder and Levine (13).

In the course of a study carried out in this Department of the part played by foetal haemoglobin in the aetiology of neonatal jaundice, some apparently unreported observations were made which seem worthy of record.

### Experimental

#### Note on Terminology Used

In view of the lack of a universally accepted nomenclature for the haem pigments, it should be pointed out that we have called the ultimate product obtained by treating haemoglobin with sodium hydroxide, alkaline globin

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haematin. This reaction involves the oxidation of the haem iron to the ferric state and the denaturation of the globin moiety. For simplicity of presentation, references to the change from haemoglobin to alkaline globin haematin will be referred to as "denaturation".

#### *Method*

The method, which is an adaptation of that of Baar and Lloyd (1), is based on the fact that, if the rate of conversion of haemoglobin to alkaline globin haematin depends on the amount of unchanged haemoglobin present at any time, then,

$$\log (H_t/H_0) = -kt$$

where  $H_0$  and  $H_t$  are the haemoglobin concentrations initially and at time  $t$  respectively, and  $k$  is the reaction constant.

The procedure used was as follows: A sample of blood was centrifuged, the plasma pipetted off, and the red cells washed twice with normal saline; 0.5 — 1.0 ml. of the red cells was introduced into approximately 100 ml. of 0.1% sodium carbonate solution. The thoroughly mixed solution was oxygenated in a Waring Blender, filtered, and its optical density measured in a Beckman Spectrophotometer. More sodium carbonate solution was then added, if necessary, to bring the optical density to a convenient value (0.90 — 1.20).

Two-milliliter samples of this solution were pipetted accurately into each of two cuvettes, to the first of which 0.4 ml. of water was added using a 1 ml. tuberculin syringe. The optical density of this solution ( $D_{H_0}$ ) was a measure of the initial haemoglobin concentration.

To the second cuvette, 0.4 ml. of  $N/4$  sodium hydroxide solution was added in a similar manner to facilitate rapid manipulation and the cuvette shaken to ensure adequate mixing. A stop watch was started at the moment of sodium hydroxide addition. The cuvette was placed immediately in the spectrophotometer and the optical density read as frequently as possible for 10 min. The readings may be represented as  $D_{H_t}$ .

At the completion of these readings, the test solution was allowed to stand for 24 hr. and its optical density  $D_B$  taken again. The calculations were made as follows:

$$\frac{H_t}{H_0} = \frac{D_{H_t} - D_B}{D_{H_0} - D_B}$$

By plotting the logarithmic ratio of the haemoglobin concentrations at the time  $t$  and initially, against the time in minutes, and extrapolating to zero time (Fig. 1), the percentages of the various haemoglobin fractions were obtained.

Preliminary experiments showed that the greatest difference in optical density between oxyhaemoglobin and alkaline globin haematin occurs at a wave length of  $578m\mu$  and our measurements were therefore made at this wave length. Baar and Lloyd used the Evelyn colorimeter with a 540 filter

but the use of the Beckman Spectrophotometer (model DU), which allows precise selection of a narrow spectral band, made it possible to obtain a sharper separation of the two pigments.

To minimize errors which might be introduced by icteric plasma, red cells centrifuged free from plasma were used throughout.

All determinations were carried out in duplicate.

#### *Preparation of a Partially Purified Sample of "Refractory" Haemoglobin*

A partially purified sample of the most refractory fraction of foetal haemoglobin was prepared so that its denaturation curve could be compared with those of cord blood and adult blood. The preparation was carried out as follows:

To 50 ml. of a 10% solution of cord blood in 0.1% sodium carbonate solution, 10 ml. of normal sodium hydroxide was added. After being allowed to stand for five minutes at room temperature, the solution was buffered by the addition of 20 ml. of normal sodium bicarbonate solution. The denatured globin fraction was then precipitated out by the addition of 80 ml. of a saturated solution of sodium sulphate. This was filtered and the clear bright red filtrate was found to contain only the most refractory fraction of foetal blood, as shown by study of its denaturation curve (Fig. 1) and the nonappearance of the characteristic band from alkaline globin haematin when examined in the Hartridge Reversion Spectroscope after the addition of ammonium hydroxide and ethyl alcohol. This was in contrast to the precipitated pigment, which, when taken up in alcohol and ammonium hydroxide, clearly showed the band in question.

#### Results

Fig. 1 shows typical denaturation curves for adult blood, cord blood, and a partially purified preparation of the most refractory component of foetal blood. While the latter takes the form of a straight line, the curve for adult blood appears as two straight lines. Since the slope of the line on the graph is a measure of the reaction constant, it may be assumed that adult blood consists of at least two forms of haemoglobin.

The situation is different, however, with cord blood (see Fig. 2 in which this curve is shown on a larger scale). In the typical example given, there is a steep slope to the one minute level; a gradual slope from the one to the three minute level, and a still more gradual slope from three minutes onwards. This would suggest that in this particular case three haemoglobins were present.

The terms "adult", "labile" or "later" haemoglobin, and "foetal" or "refractory" haemoglobin were introduced when it was believed that only two forms of haemoglobin existed. With the development of our knowledge, it is felt that these terms are not sufficiently definitive, and it is recommended that the haemoglobins of adult blood be designated  $\alpha_1$  and  $\alpha_2$ , and those of

foetal blood  $f_1$ ,  $f_2$ , and  $f_3$  (see Figs. 1 and 2). Of the components of foetal blood the fractions now designated  $f_1$  and  $f_3$  have been known for some time. Here we are concerned with the newly described form  $f_2$ .

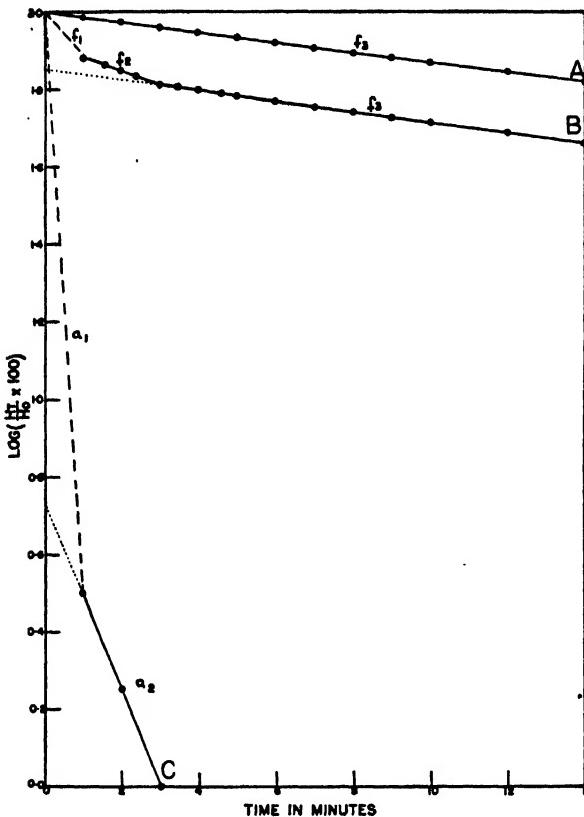


FIG. 1. Typical curves showing rate of "denaturation" of:—A, partially purified refractory haemoglobin; B, cord blood; and C, adult blood.

This form was not, however, a constant finding in our series. Of the 66 samples of infant and cord blood examined, it could be detected in only 33 cases. In the samples studied, blood was collected by syringe from the umbilical cord and by heel puncture from newborns at one, three, and seven days and again at three or four weeks after birth. All the babies were normal newborns delivered at the Winnipeg General Hospital at or near full term.

Table I shows the number of samples of cord and newborn blood at the various ages in which this third form of haemoglobin ( $f_2$ ) was apparent. It is seen from this table that it does not occur more frequently at any particular age, nor did those cases showing this third form appear to differ in any other way from those in which it was not detected.

Further light might be thrown on this problem by measurement of the reaction constants. In view, however, of the dependence of this constant on the temperature, it would be necessary to carry out all measurements at the

same constant temperature. Unfortunately, a constant temperature room or other suitable thermostatic device was not available and attempts to vary the temperature in a controlled manner led to delay in the obtaining of readings

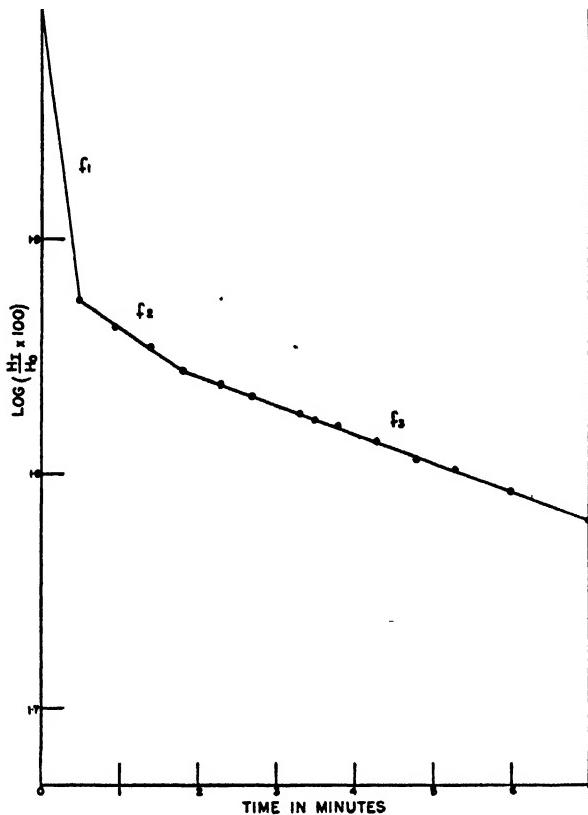


FIG. 2. Curve B, Fig. 1, drawn on a larger scale to show more clearly the distinct breaks.

TABLE I

INCIDENCE OF THE APPEARANCE OF THE INTERMEDIATE FORM OF HAEMOGLOBIN ( $f_2$ ) IN 66 CASES OF CORD AND INFANT BLOOD

	No. of cases where intermediate form apparent	No. of cases where intermediate form not apparent	Total
Cord blood	11	12	23
Babies' blood (age three days)	12	9	21
Babies' blood (age seven days)	7	11	18
Babies' blood (age three weeks)	3	1	4
	33	33	66

which was fatal to our purpose. It was found, however, that once the spectrophotometer had been allowed to warm up, the temperature of the solution to be examined remained constant throughout the course of the experiment.

### Discussion

The presence of three distinct breaks in the denaturation curves for the blood of the foetus and newborn suggests that haemoglobin may exist here in yet a third form. This form (designated  $f_3$ ) is additional to those previously described (labile,  $f_1$ , and refractory,  $a_2$ ). This new form, which is characterized by a denaturation rate intermediate between those of the other two forms, could not be detected in all of the samples studied; nor could its presence be correlated with any other factor.

So far as we are aware, the demonstration by denaturation experiments of more than two forms of haemoglobin in the blood of the foetus and newborn has not been recorded hitherto. Brinkman and Jonxis disregarded the one and two minute readings in their published curves while Ponder and Levine who were only interested in the most refractory fraction ( $f_3$ ) took their first reading 10 min. after the addition of the alkali.

It may be noted, however, that Derrien and Roche (6) have reported the isolation, by salting out methods, of three different haemoglobins from adult, and five from the blood of the newborn.

Turning now to the consideration of adult blood, the finding of two components of haemoglobin, first described by Brinkman *et al.* (4), has been confirmed by us. The possibility that the refractory components of foetal and adult blood ( $a_2$  and  $f_3$ ) might be identical was considered. If this were the case, one might expect the slopes of these two forms on the denaturation curve to be identical.

In our experiments, however, this was not the case (see Fig. 1), the refractory fraction of adult blood having a slope about twice that of the most refractory fraction of cord blood.

In summary, then, it is suggested that adult blood contains at least two haemoglobins ( $a_1$  and  $a_2$ ) and haemoglobin of the foetus and newborn may contain three fractions ( $f_1$ ,  $f_2$ , and  $f_3$ ), one of which ( $f_3$ ), however, is not always detectable. It may well be that the fractions  $a_1$  and  $f_1$  are identical as seems to be tacitly assumed by all previous workers, although this assumption still remains to be proved.

The evidence adduced here from the rate of reaction with sodium hydroxide indicates that the refractory component of adult blood ( $a_2$ ) is not identical with either of the refractory components of foetal blood ( $f_2$  and  $f_3$ ).

### Acknowledgments

We desire to express our indebtedness to Dr. H. Bruce Chown for his advice and assistance and to Dr. F. G. McGuinness and the staff of the

Department of Obstetrics and Gynaecology, Winnipeg General Hospital, for their willing co-operation.

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## THE ROLE OF THE FOETAL HAEMOGLOBINS IN THE AETIOLOGY OF JAUNDICE OF THE NEWBORN<sup>1</sup>

By G. E. DELORY, L. G. ISRAELS,<sup>2</sup> AND F. D. WHITE

### Abstract

The total bilirubin, total haemoglobin, and haemoglobin fractions were studied in the cord blood and in the heel puncture blood at one, three, and seven days, and at three or four weeks after birth, in 32 normal newborns. No relationship was found to exist between the rate of destruction of the total haemoglobin, or of its most refractory component  $f_3$ , and the degree of bilirubinaemia. The proportion of this  $f_3$  component did not always undergo progressive steady destruction, nor did it seem to be selectively destroyed in the neonatal period and it would not appear to play any special role in the production of neonatal jaundice.

### Introduction

For over 100 years, the aetiology of jaundice of the newborn has been under investigation without a clear answer forthcoming. Among the hypotheses which have been considered are: (1) increased destruction of haemoglobin due to the hyperhaemoglobinaemia occurring immediately after birth and (2) decreased elimination of bilirubin by the "immature" liver. Although only about half of all normal newborns become jaundiced after birth, the bilirubin level is always much higher than in the adult. The fact that the bilirubin concentration required to produce clinical jaundice in the newborn is considerably higher than that in the adult has been noted by many workers (11, 14) and may depend on a difference in capillary permeability or bilirubin binding power of the collagenous and elastic tissues of the skin (14). Waugh *et al.* (10) could find no absolute blood bilirubin level at which jaundice developed in all infants.

A direct relationship is, however, known to exist between the bilirubin level of the cord blood (15), the iron content of the placenta (13), and the degree and duration of the neonatal hyperbilirubinaemia. A comprehensive review of this subject has been published by Weech (11).

It has also been well known since the time of Von Kruger (9) that not only is the haemoglobin content higher in the newborn infant but that a qualitative difference also exists. As a simple illustration, when adult and cord blood are separately treated with sodium hydroxide solution, the former changes rapidly to the brown color of alkaline globin haematin while the latter may remain red for many hours. It is known that adult blood contains at least two haemoglobins and that foetal blood may contain three forms, each differing in its rate of reaction with sodium hydroxide (2, 12).

The predominant form of haemoglobin in the foetus has been loosely called "refractory" or "foetal" haemoglobin; that in the adult "labile", "adult", or "later" haemoglobin. With the increase in our knowledge, these terms are

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not sufficiently definitive and in this paper, we shall call the two pigments of adult blood  $a_1$  and  $a_2$  and those of the foetal and newborn blood  $f_1$ ,  $f_2$ , and  $f_3$ . This aspect is fully discussed by us in the preceding paper (12). All that need be said here is that the forms  $a_1$  and  $f_1$  are rapidly changed to alkaline globin haematin by the action of sodium hydroxide while  $a_2$ ,  $f_2$ , and  $f_3$  react much more slowly.

It occurred to us that it would be interesting to study the relationship (if any) between the concentrations of these pigments and the degree of neonatal hyperbilirubinaemia since it seemed possible that the development of jaundice might be due to the fact that one of these haemoglobins is more readily broken down to bilirubin.

Jonxis (5) propounded a somewhat similar hypothesis with respect to erythroblastosis. He claimed that in this condition foetal haemoglobin ( $f_3$ ) is selectively broken down almost to the exclusion of the least refractory type ( $f_1$ ). Neither Baar (1) nor Ponder and Levine (7) could confirm these observations.

So far as we know, however, no one has considered the role of the foetal haemoglobins in the aetiology of physiological jaundice.

### Methods

The babies studied were normal newborns delivered at or near full term at the Winnipeg General Hospital. Blood was collected by syringe from the umbilical cord and by heel puncture from the newborn at one, three, and seven days and again at three or four weeks, for the determination of total bilirubin, and total and differential haemoglobin. Potassium oxalate was used as the anticoagulant.

Total haemoglobin was determined by measuring the optical density of blood in a 1 in 250 dilution with 0.1% sodium carbonate solution. The measurement was made by spectrophotometric readings and standardized by iron analyses carried out according to the titanous chloride method of Delory (3).

Total bilirubin was determined by the method of Waugh (10) modified for the use of 0.1 ml. of plasma.

The degree of jaundice was assessed by inspection of the infant's skin and oral mucous membranes, and graded as follows: no jaundice —, doubtful + —, slight +, moderate ++, and severe jaundice + + +.

Estimation of the haemoglobin fraction ( $f_3$ ) was carried out by the procedure described in the preceding paper.

A recovery experiment was performed to determine the reliability of the method used to estimate the foetal haemoglobin fraction ( $f_3$ ). Haemoglobin solutions of adult and cord blood were prepared and their denaturation curves determined. The relative concentrations of the haemoglobin fractions were calculated in the usual manner. These solutions were then mixed in varying proportions, the estimation repeated, and the values obtained compared with the calculated values. The results of a typical experiment as shown in

Table I appear to afford satisfactory evidence of the reliability of the method, since the maximum difference between the experimental and calculated values was 4% of the total haemoglobin.

TABLE I

COMPARISON OF THE EXPERIMENTALLY DETERMINED VALUES OF REFRACTORY HAEMOGLOBIN ( $f_s$ ) WITH THOSE CALCULATED FROM THE DETERMINED VALUE OF 73 FOR ADULT BLOOD AND ZERO FOR CORD BLOOD

Haemoglobin mixture		Refractory haemoglobin ( $f_s$ ) as percentage of total haemoglobin	
Solution of:		Experimental value	Expected value
Cord blood	Adult blood		
10 ml.	2 ml.	61.5	61
10 ml.	5 ml.	53	49
10 ml.	10 ml.	36.5	36.5
5 ml.	10 ml.	22	24
2 ml.	10 ml.	13	12
1 ml.	10 ml.	9	7

### Results

The bilirubin, total haemoglobin, and refractory haemoglobin content of the blood was estimated in a series of 32 normal newborn infants. In the majority of cases, determinations were carried out on the cord blood, and on the third and seventh days of life, while in some cases values were also obtained at three or four weeks of age.

The results are summarized in Table II, while the complete data, including the varying degrees of jaundice, are reported in the appendix.

TABLE II

BILIRUBIN AND HAEMOGLOBIN CONCENTRATIONS IN CORD BLOOD AND BLOOD OF THE NEWBORN

	Cord blood	Third day	Seventh day	Three weeks	Four weeks
Bilirubin					
No. of cases	30	28	30	6	6
Range (mgm. per 100 ml.)	0.7-3.5	1.1-15.2	0.9-10.0	0.6-2.8	0.6-2.3
Mean (" " " ")	1.5	4.9	4.2	1.4	1.2
Total haemoglobin					
No. of cases	31	31	31	6	6
Range (gm. per 100 ml.)	11.4-20.0	14.6-22.8	14.2-22.5	15.2-20.0	10.5-19.0
Mean (" " " ")	14.9	19.2	18.2	17.3	14.9
Refractory haemoglobin					
No. of cases	32	31	32	6	6
Range (% total Hb)	50-87	45-87	44-87	57-69	40-79.5
Mean (" " " ")	76.2	76.1	76.3	64.7	70.2
Mean (gm. per 100 ml.)	11.2	14.4	13.7	11.0	10.8

### Discussion

In this series of 32 normal newborns, 19, or 59%, developed a definite clinical jaundice within the first seven days of life (cf. Appendix). This is within the range 50-75% which is generally quoted (Smith (8)) although, of course, figures such as this depend on the individual worker's concept of clinical jaundice. There was no bilirubin level (threshold) at which all infants become jaundiced but jaundice was present in all infants with a plasma bilirubin greater than 4.5 mgm. per 100 ml. The fact that the plasma bilirubin level was at its highest recorded value by the third day in the majority of the cases and had fallen by the seventh day of observation is also in agreement with the findings of other workers (15, 11). Considering now the total haemoglobin results, it will be seen that these values also rose sharply after birth and then began a sharp decline reaching the initial level in three or four weeks. Our findings of 14.9, 19.2, and 18.2 as the mean values for the total haemoglobin of cord blood and blood taken on the third and seventh days respectively, may be compared with the mean values reported by Waugh and his associates (10) of 15.36 for cord blood and 15.46 and 14.7 for the fourth and seventh days of life respectively. Faxin (4) quotes mean values of 23.2 and 21.7 for the first and third days of life, while Smith (8) found mean values of 17.9 for cord blood and 19.0 during the first week. It is clear that there is still no agreement on this important question.

With regard to the values obtained for the  $f_3$  fraction of haemoglobin, the mean value of 76.2% found by us for cord blood is in good agreement with the value of 79.75 reported by Ponder and Levine (7) for their series of 15 normal newborns. The range found by us (50-87%) differs, however, from the findings of Jonxis (6) whose values varied from 75-98%.

The steady decline in the concentration of the most refractory component as described by some authors e.g. Jonxis, was found by us to be an oversimplification of the situation. In our studies, the mean values for this fraction showed a steady increase (paralleling the total haemoglobin) up to the seventh day and then a fall to the third or fourth week at which time there was some evidence that the  $f_3$  fraction was decreasing at a greater rate than were those other components which together make up the total haemoglobin. It must be pointed out, however, that all the cases studied did not show the typical pattern suggested by the mean values.

Since from a study of our recovery experiments and duplicates, 4% of the total haemoglobin may be taken as a significant variation in the consecutive determinations of  $f_3$ , then on this basis, only 10 of the 32 infants showed a consistent decline in  $f_3$  after birth. The level in 12 infants remained the same for as long as they were followed; five newborns exhibited a rapid rise immediately after birth and five others showed an initial decline followed by a subsequent rise.

Statistical analysis revealed no correlation between the  $f_3$  content of cord blood, and the degree of bilirubinaemia developed by the infant. The correlation coefficient for this relationship was found to be 0.34 for 23 degrees of

freedom, significance at the 5% point being 0.38. Similarly no significant correlation could be established between the rate of replacement of the  $f_8$  fraction and the degree of bilirubinaemia. The correlation coefficient here was found to be 0.22 for 49 degrees of freedom. Significance at the 5% point is 0.38.

Thus no evidence could be found that the  $f_8$  haemoglobin fraction is more susceptible to destruction than the other fractions and hence it appears to be without special significance in the aetiology of neonatal jaundice.

The possible existence of a relationship between the total haemoglobin level of the blood and its subsequent rise and fall was also considered. Inspection of the results and the construction of scattergrams, however, failed to demonstrate any such relationship. The nature of the scattergrams was such that any attempt at statistical analysis was pointless, and in view of the similar conclusions of Waugh *et al.*, was not carried out.

### Acknowledgments

We desire to express our indebtedness to Dr. H. Bruce Chown, Professor of Paediatrics, for his advice and assistance, and to Dr. Fred McGuinness and the staff of the Department of Obstetrics and Gynaecology, Winnipeg General Hospital, for their willing co-operation. We are also grateful to Mr. Allan Paul of the Grain Research Laboratory, Board of Grain Commissioners of Canada, and to Mr. Barry Campbell of the Dominion Laboratory of Plant Pathology, Winnipeg, for their help with the statistical analysis.

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## APPENDIX

COMPLETE DATA OF CASES SUMMARIZED IN TABLE II

Case No.	Sex	Degree of icterus * : **		Bilirubin, mgm./100 ml.				Total haemoglobin, gm./100 ml.				Refractory haemoglobin (f), % total haemoglobin			
		3rd day	7th day	Cord blood	3rd day	7th day	3 or 4 weeks***	Cord blood	3rd day	7th day	3 or 4 weeks***	Cord blood	3rd day	7th day	3 or 4 weeks***
1	F	+ -	+	1.3	4.0	6.3	0.8	13.4	18.5	14.2	14.7	75.0	67.5	65.0	75.0
2	M	+	-	1.3	4.1	1.6	0.6	13.8	20.0	18.9	19.0	77.5	78.5	77.0	
3	M	+ +	-	0.7	2.1	1.1	0.6	15.0	20.0	22.0	19.0	50.0	45.0	44.0	40.0
4	M	-	-	0.7	2.2	2.1	(1.5)	17.5	18.0	22.3	19.5	(15.6)	82.5	83.0	(57.0)
5	M	-	-	0.7	3.6	3.3	14.5	18.5	18.1	18.1	79.5	77.5	77.5		
6	F	-	-	1.0	7.3	3.9	20.0	21.0	20.3	20.3	71.0	61.0	66.0		
7	F	+	-	1.6	3.4	1.2	(1.8)	13.8	21.0	20.0	(20.0)	69.0	66.0	55.0	(59.0)
8	F	+ -	-	1.6	3.4	1.2	(0.6)	15.2	16.8	17.7	(17.7)	74.0	68.0	83.0	(65.0)
9	M	+	+	1.0	5.9	4.3	(0.6)	19.5	19.5	15.5	(15.5)	82.5	79.5	78.5	(77.5)
10	M	-	-	1.1	1.1	1.2	(0.7)	15.5	15.5	15.5	(15.5)	80.0	68.5	75.0	(61.5)
11	M	+ + +	+ +	2.0	15.2	8.6	(2.8)	14.5	18.2	16.9	(15.2)	75.0	76.0	75.0	(69.0)
12	F	+ +	+ +	1.5	5.5	10.0	(1.0)	14.6	20.4	20.5	(18.2)	75.0	75.0	75.0	
13	M	+ +	+ +	1.5	9.0	9.0	13.0	19.0	20.5	20.5	69.0	72.0	77.5		
14	M	-	-	1.1	2.6	2.1	17.0	21.0	19.8	19.8	76.5	75.0	74.0		
15	F	+	+	3.5	9.1	6.5	17.5	17.5	17.3	17.3	82.5	82.5	83.0		
16	M	+ +	+ +	2.2	6.9	5.2	15.2	22.0	18.1	18.1	77.5	74.0	76.0		
17	M	+ +	+ +	1.8	4.2	4.3	14.7	20.1	21.4	21.4	79.5	78.5	78.5		
18	M	+ -	-	1.0	3.5	3.0	2.3	15.7	20.5	19.5	10.5	67.0	83.0	85.0	
19	M	+	+	1.6	3.4	3.4	1.0	14.9	19.6	17.2	14.9	69.0	76.0	75.0	
20	M	-	-	1.0	1.7	1.0		14.9	20.7	17.7	17.7	72.5	70.0	66.5	

• Degrees of icterus represented by:

- no jaundice.

+ - doubtful.

+ slight.

++ moderate.

+++ severe.

\*\* All cases reported were free from jaundice at birth, while of those examined at three or four weeks of age none showed any signs of icterus.

\*\*\* Figures in brackets represent values determined at three weeks, the remainder at four weeks.

## APPENDIX—Concluded

COMPLETE DATA OF CASES SUMMARIZED IN TABLE II—Concluded

Case No.	Sex	Degree of icterus . . .		Bilirubin, mgm./100 ml.		Total haemoglobin, gm./100 ml.				Refractory haemoglobin ( $f_1$ ), % total haemoglobin					
		3rd day	7th day	Cord blood	3rd day	7th day	3 or 4 weeks***	Cord blood	3rd day	7th day	3 or 4 weeks***	Cord blood	3rd day	7th day	3 or 4 weeks***
21	M	+	++	1.6	4.0	1.6	10.0	14.4	20.7	17.4		67.0	76.0	75.0	
22	M	+	++	2.0	5.4	2.0	11.4	19.8	17.5			81.0	81.0	81.0	
23	M	-	-	0.6	2.5	1.5	1.5	13.0	19.4	15.6		80.0	85.0	85.4	
24	F	+	-	1.1	7.7	2.0	2.0	12.6	14.6	16.6		87.0	87.0	87.0	
25	M	-	-	0.9	1.8	2.1	2.1	15.0	18.6	16.0		82.5	79.5	79.5	
26	M	-	+	2.0	4.1	4.1	4.1	16.0	18.6	15.0		75.5	77.0	75.5	
27	F	+ -	+-	1.1	3.8	4.3	4.3	14.3	21.5	18.5		86.0	84.0	83.0	
28	M	++	+	2.3	6.6	5.5	5.5	16.6	16.3	16.0		85.5	83.0	80.0	
29	F	-	-	1.2	2.9	0.9	0.9	15.7	21.0	16.4		16.7	78.0	78.5	
30	M	-	-	2.0	4.2	3.0	3.0	15.5	17.3	18.0		77.5	77.0	77.0	
31	M	+	++	2.4	6.2	6.2	6.2	14.1	18.2	15.6		86.5	87.0	83.0	
32	M	+	++	2.1	5.4	7.5	7.5	15.1	21.8	22.5		87.0	81.5	81.5	

\* Degrees of icterus represented by:

— no jaundice.

+- doubtful.

+ slight.

++ moderate.

+++ severe.

\*\* All cases reported were free from jaundice at birth, while of those examined at three or four weeks of age none showed any signs of icterus.

\*\*\* Figures in brackets represent values determined at three weeks, the remainder at four weeks.

## FURTHER OBSERVATIONS ON INTERFERENCE BETWEEN LYMPHOCYTIC CHORIOMENINGITIS AND MM VIRUSES<sup>1</sup>

By A. J. RHODES AND MARION CHAPMAN

### Abstract

Well marked interference is demonstrable when LCM virus is injected cerebrally in hamsters and MM virus peritoneally four or seven days later, the usual paralyzing action of the latter virus being prevented. This interference can still be demonstrated when the MM virus is injected 30 days after the LCM virus, but not when the sequence of the injections is reversed. The unparalyzed survivors of a successful interference experiment are actively immune to LCM virus. The brain, cord, and viscera of survivors, tested 10 and 11 days after the beginning of an interference experiment, contain the same amount of LCM virus as the organs of controls inoculated with this virus alone. The same organs, however, contain significantly less MM virus than the organs of controls inoculated with MM virus only. It appears that in a successful interference experiment, MM virus is prevented from multiplying in the organs of the hamster for at least six or seven days. Observations on the distribution of LCM and MM viruses in the viscera, brain, and cord of normal hamsters show that in both instances the blood is quickly invaded, and thereafter viral growth occurs in the viscera as well as the central nervous system. The reaction between the two viruses probably therefore occurs in viscera as well as central nervous system.

### Introduction

We have already drawn attention to the interference phenomenon that can be demonstrated in hamsters inoculated with the antigenically unrelated lymphocytic choriomeningitis (LCM) and MM viruses (7). When the LCM virus is injected cerebrally, and the MM virus peritoneally four to seven days later, a significant number of the animals fails to develop paralysis due to MM virus. This reaction appeared worthy of further study as a model of the type of interference that occurs between unrelated viruses inoculated by different routes. We now report on our study of three aspects of this phenomenon: (1) An investigation of the distribution of the viruses in the organs of normal hamsters. (2) A study of the distribution of the viruses in hamsters surviving an interference experiment. (3) An examination of the relationship between interference and active immunity. The results of these studies will be reported in three sections, and will then be discussed together in the final section.

### I. The Distribution of LCM and MM Viruses in Normal Hamsters

#### THE DISTRIBUTION OF LCM VIRUS IN HAMSTERS INOCULATED CEREBRALLY

This study, and the parallel one with MM virus, was carried out with a view to determining, if possible, the organs that might be involved in interference. Incidental to this study, observations were made on the pathogenesis of these infections in hamsters.

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Contribution from Connaught Medical Research Laboratories, University of Toronto, Toronto, Ont. Aided by a grant from the National Foundation for Infantile Paralysis.

### Methods

The details of preparation of the pools of LCM virus used in the present study were given in our earlier report, and remained the same in this investigation.

A group of hamsters 21 to 24 days old was inoculated cerebrally with 0.05 ml. of a  $10^{-1.7}$  dilution of LCM infected mouse brain, a dose previously found to cause recognizable signs of infection in all hamsters of this age. At stated intervals after inoculation, single animals were anesthetized, and 1 to 2 ml. of heart blood withdrawn and treated with heparin. The animal was then perfused through the heart with 50 to 60 ml. of 0.1% heparin in saline. The liver, spleen, kidney, brain, and cord were removed and stored in the dry-ice box. Subsequently, dilutions of whole blood, and suspensions of the solid organs were prepared; serial 10-fold dilutions were inoculated cerebrally into groups of six 12 to 14 gm. mice in 0.03 ml. amounts, in order to establish the approximate virus content of the various organs. Deaths of mice were recorded and the  $LD_{50}$  levels were determined by the method of Reed and Muench customarily used in this type of work.

### Results

As can be seen from Table I, LCM virus was first demonstrated in the blood 24 hr. after cerebral inoculation and viraemia continued throughout the 12 day period of the experiment.

TABLE I

TITRATION OF HAMSTER ORGANS HARVESTED AT INTERVALS AFTER CEREBRAL INOCULATION OF LCM VIRUS

Interval after inoculation	Titer of virus in terms of $LD_{50}$ for mice		
	Blood	Viscera	Brain and cord
<b>Hours</b>			
2	Nil		
6	Nil	$10^{-1.0}$	$10^{-1.0}$
12	Nil		$10^{-1.5}$
24	$10^{-1.0}$	$10^{-2.7}$	$10^{-1.4}$
36	$10^{-2.5}$		$10^{-4.5}$
48	$>10^{-2.0}*$	$10^{-4.0}$	$10^{-6.0}$
60	$10^{-3.2}$		$>10^{-5.5}*$
72	$>10^{-5.0}*$		$>10^{-6.0}*$
84	$>10^{-6.0}*$	$>10^{-5.0}*$	$>10^{-6.0}*$
96	$10^{-4.3}$	$>10^{-6.0}*$	$>10^{-6.0}*$
<b>Days</b>			
5			$10^{-6.0}$
6	$10^{-5.4}$	$10^{-6.3}$	$10^{-6.7}$
7	$>10^{-6.0}*$		$10^{-6.5}$
8	$10^{-5.4}$	$>10^{-6.5}*$	$10^{-6.4}$
9			$>10^{-6.0}*$
10	$10^{-5.0}$	$10^{-6.3}$	$10^{-6.4}$
11			$>10^{-6.0}*$
12	$10^{-5.3}$	$10^{-5.7}$	$10^{-6.3}$

\* End points were not reached in these titrations, owing to the unexpectedly large amounts of virus present.

Virus was recovered from the pooled viscera as early as six hours, presumably having been transported there from the site of inoculation by the blood stream, although at this early stage no virus was actually recovered from the blood. Large quantities of virus persisted for at least 12 days.

In the brain and cord, viral multiplication began between 24 and 36 hr. after inoculation, maximal quantities of virus being found between two and five days after inoculation; large amounts were still present 12 days after inoculation.

### THE DISTRIBUTION OF MM VIRUS IN HAMSTERS INOCULATED PERITONEALLY

#### Methods

MM virus pools were prepared as previously described. A group of hamsters 21-24 days old was inoculated peritoneally with 0.5 ml. of a  $10^{-2}$  dilution of infected mouse brain, a dose known to paralyze practically all animals of this age. At frequent intervals after inoculation, hamsters were sacrificed, and the organs removed following the procedure used for the animals infected with LCM virus.

#### Results

The results are given in Table II, from which it will be seen that MM virus rapidly entered the blood, being demonstrable as early as four hours. Large amounts of virus circulated from 20 to 36 hr. after inoculation and throughout the duration of the experiment.

TABLE II

TITRATION OF HAMSTER ORGANS HARVESTED AT INTERVALS AFTER PERITONEAL INOCULATION OF MM VIRUS.

Interval after inoculation, hr.	Titer of virus in terms of LD <sub>50</sub> for mice		
	Blood	Viscera	Brain and cord
2	Nil		
4	$10^{-4.5}$	$10^{-3.3}$	
6		$10^{-4.0}$	
8	$10^{-8.0}$		
12	$10^{-3.2}$	$10^{-4.4}$	$10^{-1.7}$
16	$10^{-3.0}$		$10^{-4.2}$
20	$>10^{-5.0}*$		$10^{-4.4}$
24	$>10^{-5.0}*$	$10^{-4.4}$	$10^{-5.3}$
30		$10^{-4.0}$	
36	$>10^{-5.0}*$	$10^{-4.8}$	$10^{-6.4}$
42	$10^{-4.2}$	$10^{-4.5}$	$10^{-5.2}$
48	$10^{-3.5}$	$10^{-3.7}$	$10^{-5.6}$
54	$10^{-4.1}$		$10^{-5.5}$
60	$10^{-4.2}$	$10^{-4.7}$	$10^{-6.0}$
72	$10^{-4.5}$	$10^{-4.0}$	$10^{-6.0}$

\*End points were not reached in these titrations, owing to the unexpectedly large amounts of virus present.

In the pooled viscera, the amount of virus remained fairly high and relatively constant from shortly after inoculation till the conclusion of the experiment.

In the brain and cord, only a small amount of virus was present at 12 hr. after peritoneal inoculation. Between 16 and 36 hr., there was a marked increase in the amount of virus, which remained relatively constant till the end of the experiment.

## II. The Distribution of LCM and MM Viruses in Survivors of Interference Experiments

### Methods

This study was carried out using the survivors of an interference experiment referred to in our previous report as Experiment 4; the interval between the inoculation of LCM and MM viruses was four days, and 9/10 animals failed to develop paralysis, whereas only 2/10 controls failed to become paralyzed.

Two unparalyzed hamsters were sacrificed 10 days after the start of the experiment, and two 11 days after the start, that is to say respectively six and seven days after the inoculation of MM virus. Appropriate controls, inoculated with LCM or MM viruses only were likewise sacrificed. The brain, cord, and viscera were removed and stored frozen. Later, suspensions of these organs were prepared in broth, serial dilutions made, and inoculated cerebrally in groups of six to eight mice (0.03 ml. amounts). In no case were less than four serial 10-fold dilutions tested. The animals were closely examined, and the day of death recorded. Death occurring within five days was regarded as caused by MM virus, whereas sickness developing from the sixth day on was attributed to LCM virus. This method, of course, demonstrated the presence of MM virus rather than LCM virus, for mice inoculated with a mixture of these viruses died from the short incubation MM illness rather than the more slowly progressive LCM infection. However, as the MM virus content of the organs of survivors was very low, it is believed that the method also estimated reasonably accurately the amount of LCM virus in these organ suspensions.

Reference to Table III, which gives details of the time of death of the various mice inoculated in this experiment, shows that when serial dilutions of organs known to contain MM virus alone were injected, nearly 90% of the mice died by the end of the fifth day. In the same five day period only about 3% of mice injected with suspensions of organs containing LCM virus died. It appears, therefore, that our criteria for apportioning the cause of death in the mice inoculated with organ suspensions which might contain both viruses were acceptable.

### Results

The concentrations of the viruses in the organs of hamsters sacrificed 10 days after the start of the experiment are given in Table IV. It will be seen that

TABLE III  
RESULT OF INOCULATION OF MICE WITH DILUTIONS OF INFECTED HAMSTER ORGANS

	Number of mice dying	Number of mice dying by end of fifth day	Deaths beyond five days
Organs from hamsters receiving MM virus only	201	173 (86.6%)	28 (13.4%)
Organs from hamsters receiving LCM virus only	356	12 (3.4%)	344 (96.6%)
Organs from hamsters inoculated with both viruses	366	14 (3.8%)	352 (96.2%)

TABLE IV  
DISTRIBUTION OF MM AND LCM VIRUSES IN ORGANS OF HAMSTERS SACRIFICED 10 DAYS AFTER BEGINNING OF EXPERIMENT

Hamster No.	State as regards inoculation	Approximate content of LCM virus in:			Approximate content of MM virus in:		
		Viscera	Brain	Cord	Viscera	Brain	Cord
1	Controls: MM virus only				$10^{-4.8}$ $10^{-4.2}$	$10^{-4.2}$ $10^{-4.5}$	$10^{-3.6}$ $10^{-4.0}$
2							
3	Controls: LCM virus only	$>10^{-3.5}$ $>10^{-3.6}$	$>10^{-5.5}$ $>10^{-5.5}$	$>10^{-5.5}$ $>10^{-5.5}$			
4							
5	Survivors of interference experiment	$10^{-4.2}$ $10^{-3.5}$	$>10^{-5.5}$ $>10^{-5.5}$	$>10^{-5.5}$ $>10^{-5.5}$	$<10^{-1.0*}$ $10^{-1.3}$	$<10^{-1.0*}$ $<10^{-1.0*}$	$<10^{-1.0*}$ $<10^{-1.0*}$
6							

\* Indicates no virus detected in  $10^{-1}$  dilution of organs.

considerable quantities of MM virus were found in the organs of controls. By comparison, no MM virus was recovered from 1:10 suspensions of the brain or cord of the survivors of the interference experiment, and was found in small amount in the viscera of one animal only. LCM virus was found in essentially the same quantities in the organs of survivors and controls.

The concentrations of the viruses in the organs of hamsters sacrificed a day later, i.e. 11 days after the beginning, were as shown in Table V. These results are in agreement with those given in Table IV, the content of MM virus being definitely less in the survivors of the interference experiment than in the controls inoculated with MM only.

TABLE V

DISTRIBUTION OF MM AND LCM VIRUSES IN ORGANS OF HAMSTERS SACRIFICED 11 DAYS AFTER BEGINNING OF EXPERIMENT

Hamster No.	State as regards inoculation	Approximate content of LCM virus in:			Approximate content of MM virus in:		
		Viscera	Brain	Cord	Viscera	Brain	Cord
1	Controls: MM virus only				$10^{-1.7}$	$10^{-2.4}$	$10^{-2.8}$
2					$10^{-1.8}$	$10^{-2.6}$	$10^{-3.1}$
3					$10^{-1.8}$	$10^{-2.6}$	$10^{-3.4}$
4					$10^{-1.8}$	$10^{-2.6}$	$10^{-4.0}$
5	Controls: LCM virus only	$>10^{-2.5}$	$>10^{-2.5}$	$>10^{-2.5}$			
6		$>10^{-2.5}$	$>10^{-2.5}$	$>10^{-2.5}$			
7	Survivors of interference experiment	$>10^{-2.5}$	$>10^{-2.5}$	$>10^{-2.5}$	$<10^{-1.0*}$	$<10^{-1.0*}$	$<10^{-3.0**}$
8		$>10^{-2.5}$	$>10^{-2.5}$	$>10^{-2.5}$	$<10^{-1.0*}$	$<10^{-1.0*}$	$<10^{-2.0**}$

\* Indicates no virus detected in  $10^{-1}$  dilution of organs.

\*\* Indicates no virus detected in  $10^{-2}$  dilution, which was the most concentrated tested.

### III. Relationship Between Interference and Active Immunity

#### Susceptibility of New Strain of Hamster

It became necessary at this stage to change the source of supply of hamster from a local breeder to Tumblebrook Farms, and accordingly the susceptibility to MM virus of the two strains of golden hamster was compared. As shown in Table VI, young Tumblebrook hamsters, three to four weeks old, proved

TABLE VI  
SUSCEPTIBILITY OF TWO STRAINS OF GOLDEN HAMSTER TO MM VIRUS

Source of hamster (aged 3-4 weeks)	Incidence of paralysis following intraperitoneal inoculation of dilutions of MM virus	
	$10^{-2}$	$10^{-3}$
Local	25/33 (76%)	22/33 (66%)
Tumblebrook Farms	36/38 (95%)	17/18 (94%)

somewhat more susceptible to MM virus than our local hamsters. Several satisfactory interference experiments have been conducted with the new strain which is eminently suitable for this work.

#### Interference Experiment

In order to have available for study a number of survivors, a further interference experiment was carried out along the usual lines but using the Tumblebrook Farms hamster. The results are shown in Table VII, from which it will

TABLE VII  
DETAILS OF INTERFERENCE EXPERIMENT IN HAMSTERS

Interval between primary injection of LCM and inoculation of MM virus	Dilution of LCM virus in form of mouse brain (0.05 ml. amounts)	Dilution of MM virus in form of mouse brain (0.5 ml. amounts)	Numbers of animals that developed paralysis on inoculation with MM	
			Treated with LCM	Controls, untreated with LCM
4 days	10 <sup>-2</sup>	10 <sup>-1</sup>	1/30*	18/20*

\* These differences are highly significant.

be evident that of 30 animals receiving both viruses, only one became paralyzed so that 29 survivors were available.

#### Cross-Resistance Tests

These 29 survivors were held for 30 days, and were then divided into groups and challenged by the cerebral route with either LCM or MM virus (0.05 ml. of a 10<sup>-1</sup> suspension). Appropriate control groups of animals of the same age at the time of challenge, but previously uninoculated with either virus, were injected at the same time. The results of challenge with MM virus are given in Table VIII and with LCM virus in Table IX.

TABLE VIII

**RESULTS OF CEREBRAL CHALLENGE WITH MM VIRUS IN HAMSTERS SURVIVING INTERFERENCE EXPERIMENT**

Description of animals challenged	Number of animals challenged*	Results of challenge
Normal controls of same age as survivors	10	10/10 became paralyzed
Inoculated with MM only at time of interference experiment; convalescent from paralysis	9	None showed any extension of paralysis
Unparalyzed survivors of interference experiment	14	None became paralyzed
Inoculated with LCM only at time of interference experiment	6	Only 1/6 developed transient paralysis

\* Challenge carried out 30 days from start of original interference experiment.

From Table VIII it will be seen that when MM virus was given cerebrally to normal controls of the same age as the survivors, all became paralyzed. The animals inoculated with MM only at the time of the interference experiment and convalescent from their paralytic attacks, showed no extension of

TABLE IX

## RESULTS OF CEREBRAL CHALLENGE WITH LCM VIRUS IN HAMSTERS SURVIVING INTERFERENCE EXPERIMENT

Description of animals challenged	Number of animals challenged*	Results of challenge
Normal controls of same age as survivors	10	Only 3/10 became sick.
Inoculated with LCM only at time of interference experiment	6	1 died within 7 days; remainder healthy
Inoculated with MM only at time of interference experiment; convalescent from paralysis	9	1 died within 7 days; 4 showed signs of mild LCM infection; 4 remained <i>in statu quo</i>
Unparalyzed survivors of interference experiment	13	None developed any sickness attributable to the challenge

\* Challenge carried out 30 days from start of original interference experiment.

paralysis on challenge and had presumably become actively immune. None of 14 survivors of the interference experiment developed paralysis when challenged with MM. This might be interpreted as due to active immunity, but consideration must be given to the fact that of six animals inoculated with LCM virus alone at the time of the interference experiment only one became paralyzed when challenged with MM 30 days later. It would appear therefore that the blocking action of LCM against MM virus persists for at least 30 days.

The interpretation of the results shown in Table IX is rendered difficult by the fact that the preparation of LCM virus, although fully potent for the young animals used at the start of the experiment, was of reduced virulence for the animals 30 days older, only 3/10 of which were affected clinically. Nevertheless, it would appear that the 13 survivors of the interference experiment were actively immune to challenge with LCM virus, as they developed no further sickness. It is of interest to note that the nine animals originally inoculated with MM virus alone showed no resistance to the LCM challenge. There does not therefore appear to be any interference demonstrable over the 30 day period when the MM virus is given first.

#### IV. Discussion

The problem of interference between animal viruses has been much studied of recent years, and Findlay (4) and Henle (6) have both recently reviewed the question. In our study of the problem we have investigated the blocking action of lymphocytic choriomeningitis (LCM) virus injected cerebrally in hamsters against the MM virus injected peritoneally, as first described by Dalldorf and Whitney (3). This interference is evidenced by the sparing from MM paralysis noted in these animals previously inoculated with LCM virus.

A similar sparing effect from clinical signs of infection or from death has been demonstrated with several other pairs of antigenically unrelated animal viruses. For example, when Rift Valley fever virus is inoculated intraperitoneally in rhesus monkeys, some of the animals are protected against the effects of an inoculation of yellow fever virus given shortly afterwards (5). To give another example, mice injected cerebrally with influenza virus withstand an injection by the same route of the unrelated Western equine encephalomyelitis virus (9). In this type of interference, it is usual for the interfering (blocking) virus to cause a slowly progressive infection which by itself proves lethal to only a small percentage of animals. The blocked virus on the contrary is one that causes a rapidly progressive, commonly fatal infection in control animals.

Of particular interest in the study of interference between such viruses is the actual fate of the virus which appears to be blocked. Some such investigations have been made. For example, Dalldorf (2) studied the interference that can be demonstrated in monkeys injected cerebrally with LCM and the MV strain of human poliomyelitis virus; he found that the sparing effect was associated with an absence of an infectious amount of MV virus in the cervical enlargement of the spinal cord. Similar observations were made by Vilches and Hirst, the growth of Western equine encephalomyelitis virus being completely suppressed by influenza virus (9). A different site of interaction was studied by Andrewes (1) who injected mixtures of virus III and fibroma viruses intradermally in rabbits. Virus III suppressed completely the development of fibromata, and the rabbits did not become resistant to fibroma virus. These three observations concern interference between unrelated viruses injected into an animal by the same route. In the reaction that we have studied, the situation is more complicated because the viruses are inoculated by different routes, the MM virus reaching the central nervous system presumably by the blood stream.

In studies on the distribution of LCM and MM viruses in normal hamsters we found that after cerebral inoculation, LCM virus rapidly invades the rest of the body, being found in the blood and viscera before it starts to multiply in the brain and cord. At the critical period of four to seven days after inoculation, when MM virus is inoculated in interference experiments, large amounts of LCM virus are present in the brain, cord, viscera, and blood. Following peritoneal inoculation, MM virus can be recovered from the blood and viscera after four hours. By 16 hr., multiplication is proceeding in the brain and cord. Virus can be recovered in large amount from blood, viscera, and central nervous system for at least 72 hr.

It is evident, therefore, that both LCM and MM viruses proliferate in the viscera as well as the central nervous system of hamsters, and they can be regarded as showing both viscerotropism and neurotropic properties. Incidentally, it is of interest to note that large amounts of either virus can be recovered from organs substantially before there are any recognizable signs of sickness.

In studies on the distribution of LCM and MM viruses in survivors of interference experiments it was shown that LCM virus proliferates in the viscera, brain, and cord to the same extent as in controls. The state of affairs with MM virus was, however, found to be markedly different, for the virus was not detected at all in the brain or cord of three of the four animals tested and only in low concentration in the viscera of one of the animals. More extensive tests, which we are not at the moment in a position to perform, should be carried out to determine whether this initial suppression of MM virus growth continues in animals sacrificed at a later stage of the experiment. One would expect the same conditions to prevail, as clinical observation of surviving hamsters over a four week period has shown no tendency to the development of MM paralysis after the sixth or seventh day following the inoculation of this virus. Our results are therefore in agreement with those of workers using other pairs of viruses who demonstrated by infectivity tests that growth of the blocked virus was in fact markedly inhibited. Our studies bring forward the additional observation that inhibition of growth occurs not only in the brain and cord but also in the viscera. It is probable that a very considerable number of host cells must be altered by the LCM virus in some way that prevents multiplication of the MM virus, during the critical period after inoculation of the second (MM) virus. It would seem reasonable to suggest that the reason why interference cannot be demonstrated when the LCM and MM viruses are injected 48 hr. apart (7) is that at that early stage not enough multiplication of LCM virus has occurred to alter the susceptibility of the cells available to the MM virus.

Another aspect of the interference phenomenon that interested us was relationship to immunity. We found that unparalyzed survivors do not develop any sickness attributable to a challenge of LCM virus given 30 days after the beginning of the experiment, so that active immunity to the blocking virus probably develops, as one might expect from the fact that this virus is uninhibited in its growth. Survivors also resist a challenge of MM virus given 30 days after the beginning of the experiment. However, one cannot state definitely that this resistance is due to the development of active immunity, because animals inoculated in the first instance with LCM virus alone are likewise resistant to MM virus 30 days later. The probable explanation is that LCM virus survives in the brain and probably viscera for this long period after direct cerebral inoculation, and that interference is associated with the continued presence of active LCM virus.

The finding that interference can still occur after 30 days is of considerable general interest, because a not uncommon method of investigating the antigenic relationship of two viruses is to challenge immunized animals. Before suggesting, therefore, that cross-resistance between two viruses implies a common antigenic structure, it is necessary to exclude the possibility that resistance is due to interference set up by virus persisting from the original immunizing inoculations. In this connection, one may recall the observations of Schlesinger, Olitsky, and Morgan (8) relating to the same type of problem. These workers

found that guinea pigs rendered resistant to Western equine encephalomyelitis virus by vaccination and by cerebral injection resisted cerebral injection of the antigenically distinct Eastern equine encephalomyelitis and vesicular stomatitis viruses.

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## **NOTICE**

The Canadian Journal of Research is at present published in six sections, A to F. Starting with January 1, 1951, these sections will be published as separate journals under distinctive names and the designation Canadian Journal of Research will no longer be used. The present names and the corresponding new names are as follows:

<b>PRESENT NAME</b>	<b>NEW NAME</b>
Canadian Journal of Research, Section A (Physical Sciences)	Canadian Journal of Physics
Canadian Journal of Research, Section B (Chemical Sciences)	Canadian Journal of Chemistry
Canadian Journal of Research, Section C (Botanical Sciences)	Canadian Journal of Botany
Canadian Journal of Research, Section D (Zoological Sciences)	Canadian Journal of Zoology
Canadian Journal of Research, Section E (Medical Sciences)	Canadian Journal of Medical Sciences
Canadian Journal of Research, Section F (Technological Sciences)	Canadian Journal of Technology

In order to preserve continuity the present sequence of volume numbers will be retained, and in each case the volume for 1951 will be Volume 29.

The subscription rates for the Journals will remain as at present.



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## VIBRIO CHOLERAE IN FLUID MEDIA<sup>1</sup>

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### Abstract

Studies have been made of the growth of *V. cholerae* in fluid media of chemically defined compositions. The addition of three amino acids, tyrosine, asparagine, and glycine, to a fluid medium containing inorganic salts produced a growth of *V. cholerae* equivalent to a 450 p.p.m. silica standard. Under conditions of aeration with an air and carbon dioxide mixture, yields comparable to the turbidity of a 1600 p.p.m. silica standard were obtained with a medium composed of 0.67 gm. of tyrosine, 0.42 gm. of asparagine, 0.51 gm. of glycine, 5.0 gm. of sodium chloride, 5.0 gm. of ammonium sulphate, 0.75 gm. of dipotassium hydrogen phosphate, 0.1 gm. of magnesium sulphate, 10.0 gm. of glucose, and 15.0 gm. of sodium bicarbonate dissolved in one liter of distilled water.

As cholera has not invaded this continent since 1873, the demand for cholera vaccine in Canada is ordinarily small. It is required only to satisfy the needs of travellers into cholera endemic areas in the Near and Far East, where the disease still counts its victims in the hundreds of thousands every year. In view of the existence of such a nidus, there is always a possibility that large quantities of cholera vaccine may suddenly be required, even in Canada, to immunize a threatened or invaded population.

The method for the production of cholera vaccine as employed in Canada was reported by Ranta and Dolman (8) in 1943. In this method, selected strains of *V. cholerae* are grown on solid medium and harvested from it with phenolized saline. Production in this way necessitates considerable handling of materials in relatively small batches, and large-scale production becomes cumbersome.

In 1944, Linton and Jennings (5, 2, 3) reported on a procedure for the development of a heavily turbid growth of *V. cholerae* in a buffered casein digest fluid medium, in which the yield had been enhanced by aeration with a carbon dioxide and air mixture. Though Linton and Jennings were primarily interested in a heavy yield for their biochemical studies of the vibrios, it did not

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escape them that this method might be adapted for vaccine production. But its value as a means for producing an effective vaccine was apparently not tested.

As inoculation with digested proteins may result in local and, rarely, generalized reactions, safety demanded the use of the simplest medium that would support an abundant growth of *V. cholerae* in a satisfactorily antigenic form. Moreover, any newly developed cholera vaccine must compete with the present vaccine, which is probably the most benign product among the active immunizing agents. These considerations led to an investigation to determine the results of growth of *V. cholerae* in chemically defined fluid media. The work of Koser and Rettger (4) in 1919, and Robertson (10) in 1924, on the nutrition of *V. cholerae*, and the fact that peptone water alone supports ample growth, encouraged the search for a simple synthetic medium.

### Experimental Procedures

*V. cholerae*, Strain No. 41 (Ogawa) was used throughout the study. All media were adjusted to pH 8.5 before sterilization, and to effect rapid and uniformly dispersed growth, instead of a surface pellicle, provision was made for the aerobic utilization of a simple sugar. The mineral demands were satisfied by a basic medium containing 5.0 gm. of sodium chloride, 0.75 gm. of dipotassium hydrogen phosphate, and 0.1 gm. of magnesium sulphate, in one liter of distilled water. When this was put to use, amino acids and glucose were added, the latter in quantities of 0.1, 0.2, and 0.3%. As growth occurred in the basic mineral medium when an enzymatic digest of casein was added, it was decided to incorporate amino acids into the basic medium in the percentages reported for casein. The fallacy of this conclusion was fully appreciated, but the procedure was meant to serve merely as a starting point. Accordingly, appropriate concentrations of 20 amino acids were made up in 50 ml. quantities.

In selecting the amino acids for study, some guidance was also afforded by the work of Mitra (6, 7) in 1936, who reported on the racemization of the proteins of *V. cholerae*. Thus, special attention was paid to the 12 amino acids that he identified: glycine, alanine, valine, leucine, tyrosine, aspartic acid, glutamic acid, proline, hydroxyproline, arginine, histidine, and lysine.

The basic medium, with amino acids added, was dispensed in 5 ml. quantities into large test tubes. During incubation the tubes were slanted as much as practicable in order to facilitate aeration. This medium was seeded with 0.4 ml. of a culture grown in casein digest basic medium, but the effect of the small quantity of casein digest transferred in the inoculum was obliterated by four serial transfers in each amino acid basic medium at 24 hr. intervals. As a control, each series of tests was accompanied by an inoculated tube of casein digest basic medium.

Observations were made at intervals up to 64 hr. after inoculation, and the growth, if any, was compared with silica standards (1), ranging in steps of 50 p.p.m. from 100 to 1000 p.p.m.

At first, each amino acid was tested separately. Then, pairs of amino acids were tried, but it was not necessary to use more than 45 of the 190 possible paired combinations, for omissions were indicated when experiments in this paired series showed a member of a pair to be inhibitory in its action upon *V. cholerae*. For further study, single amino acids were added to pairs that had given the most promising growth. Later, certain pairs of amino acids were similarly added to other pairs, so that the greatest number in any of the tested media did not exceed four amino acids.

When a suitable combination of amino acids was found, *V. cholerae* was grown in liter quantities of the medium after some further mineral salts were added.

During the period of growth these cultures were subjected to continuous aeration. This was carried out by passing air from a compressed air tank through a calibrated glass flowmeter. Before reaching the flowmeter, the gas travelled through the horizontal arm of a T-shaped glass tube, the vertical arm extending downward about one meter. The open end of the vertical arm of the T-tube was immersed in water contained in a tall cylinder, so that the pressure of nearly one meter of water prevented the air from bubbling into the cylinder, but if the gas pressure suddenly surged, the apparatus acted as a satisfactory safety device. A similar appliance served to pass carbon dioxide from a cylinder through a flowmeter. Using a light oil in the U-tubes of the flowmeters, the delivery was adjusted to provide 15 liters of air and 5 liters of carbon dioxide per hour.

The tubes leaving the flowmeters passed the gases through chambers containing sterile cotton wool; then, by a common tube, the gases were led into the incubator and into the depths of a three-necked, 2-liter distilling flask containing one liter of medium. The gases were dispersed into the medium by tying a fine linen bag over the flared end of a glass tube. One of the necks of the flask was fitted with a wide-bored glass tube. This reached just below the surface of the medium. By means of a rubber bulb, the medium could be drawn periodically into the tube in order to compare the turbidity of the growth with silica standard suspensions kept in similar tubes. The third neck was stoppered with cotton wool to permit escape of the aerating gases.

### Growth in Nonaerated Amino Acid Basic Media

On the whole, the addition of a single amino acid to the basic medium of mineral salts resulted in sparse growth of *V. cholerae*. After incubation for 24 hr. in the fourth subculture, the best results were obtained with asparagine, the acid amide of aspartic acid. The turbidity equalled that of a 200 p.p.m. silica standard, while casein digest basic medium produced a turbidity of 250 p.p.m. Traces of growth were supported by alanine, valine,

serine, phenylalanine, hydroxyproline, lysine, and arginine; while turbidities up to 100 p.p.m. were obtained with glycine and with isoleucine. Many amino acids supported no growth: *viz.*, histidine, methionine, proline, leucine, tryptophane, cystine, aspartic acid, glutamic acid, and threonine.

Of the pairs of amino acids, tyrosine and glycine produced a density of growth equalling 250 p.p.m., and corresponding to the growth in casein digest basic medium. Tyrosine and isoleucine gave a growth of 200 p.p.m., and tyrosine and alanine developed a turbidity of 175 p.p.m. Only one other amino acid seemed to encourage growth. While the inhibition caused by aspartic acid made it unsuitable, asparagine in combination with tyrosine, histidine, or with glycine produced a growth of either 125 or 150 p.p.m. Thus, the stimulating effect of asparagine, when used alone, was maintained when it was added to some other amino acids. This was not a general rule, for a combination of amino acids with tyrosine fostered the greatest yield, despite its inhibitory action when used alone.

In tests of trios of amino acids, the turbidities obtained upon the addition of an amino acid to combined tyrosine and glycine rarely resulted in an increased yield. Tryptophane and proline both completely inhibited the growth, and several other amino acids, such as, aspartic acid, cystine, hydroxyproline, threonine, methionine, phenylalanine, serine, valine, and histidine, reduced the yield to less than 200 p.p.m. The addition of alanine, isoleucine, or of lysine brought about yields of 300 p.p.m., while arginine increased the density to 375 p.p.m. But asparagine brought about the greatest yield. Instead of 250 p.p.m. as obtained with combined tyrosine and glycine, the addition of asparagine produced a density of 450 p.p.m., or roughly double the yield in casein digest basic medium.

As the yield in tyrosine - asparagine - glycine basic medium (TAG medium) was encouraging, the remainder of this part of the investigation consisted of adding single amino acids to TAG medium. In no case was the yield increased by the addition of an amino acid. In fact, the general response was an appreciable reduction in turbidity to between 125 and 225 p.p.m.

### Growth in Aerated Buffered Media

As the results obtained with nonaerated TAG medium gave promise of a reasonably large yield, it was tested by employing aeration and the carbon dioxide/sodium bicarbonate buffering system recommended by Jennings and Linton (3).

For aeration, TAG medium was prepared as follows: 0.67 gm. of tyrosine, 0.51 gm. of glycine, 5.0 gm. of sodium chloride, 5.0 gm. of ammonium sulphate, 0.75 gm. of dipotassium hydrogen phosphate, and 0.1 gm. of magnesium sulphate were dissolved in one liter of distilled water. The pH was adjusted to 8.5 before sterilization in an autoclave at 15 lb. pressure for 20 min. Before inoculation, the following substances were added: 0.42 gm. of asparagine, autoclaved separately in dry form; 10.0 gm. of glucose, autoclaved

separately in a minimum of distilled water; and 15.0 gm. of sodium bicarbonate autoclaved in dry form. Carbon dioxide from a pressure tank was bubbled through the medium for a few minutes before it was inoculated with 100 ml. of an overnight culture of *V. cholerae* in unaerated TAG medium.

The yields after 48 hr. growth in buffered, aerated TAG medium have consistently been over 650 p.p.m., with the majority ranging over 1000 p.p.m., and reaching as high as 1600 p.p.m.

Studies to modify the amino acid concentrations of TAG medium have shown that no material changes are obtained by doubling the concentration of any one, or all of the amino acid constituents, although increases in asparagine reduced the density of growth by approximately one-half. The substitution of the amino acids in TAG medium has shown that comparable yields can be obtained by replacing the asparagine and glycine with 0.97 gm. of isoleucine, the tyrosine with 0.16 gm. of alanine, or the asparagine with 0.39 gm. of arginine.

### Discussion

The greatest yields obtained with TAG medium were equivalent to a 1600 p.p.m. silica standard. This density is about thrice greater than the density of cholera vaccine now employed for human immunization. Samples of these and other experimental lots have been preserved by diluting with physiological saline to produce a final concentration of 8000 million *V. cholerae* per ml. The organisms in these suspensions have been killed by the addition of phenol to a final concentration of 0.5%. The vaccines, so prepared, are at present being compared antigenically with the standard type of cholera vaccine, employing the mouse protection test reported by Ranta and Dolman (9) in 1944.

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## PARALLEL STUDIES OF COMPLEMENT AND COAGULATION<sup>1</sup>

### I. THE EFFECT OF VITAMIN C

By CHRISTINE E. RICE AND PAUL BOULANGER

#### Abstract

Groups of guinea pigs on a basic diet of commercial rabbit pellets supplemented by fresh green grass or stored roots exhibited marked differences in the ascorbic acid level of their plasma. These differences were not accompanied by any significant variation either in the complement titer of the serum or the prothrombin time of the plasma. Such minor variations as were observed in these two activities did not parallel each other.

#### Introduction

The question of whether the two important normal properties of the blood, complement and coagulative activity, are basically related has interested physiologists and immunologists for many years (4, 6-10, 15-18, 27, 28). Aside from the fact that both phenomena involve the precipitation of blood proteins, the major evidence suggesting the possible relationship of the two reactions has been that they are affected by many of the same factors *in vitro*, while *in vivo* a decrease in both activities has been observed in certain pathological conditions, notably in liver disease and in anaphylactic shock. On the other hand, data indicating that the relative amounts of the various reagents required to inhibit the two reactions may vary widely, and the further observation that the coagulation time of the blood may increase without an accompanying decrease in complement titer, has suggested to other groups of investigators that the two reactions are independent of each other.

Both complement activity and coagulation are, however, complex phenomena involving a series of components, all of which are essential for the completion of the respective reactions. The complement complex consists of at least four major components, two of which are globulins, one apparently a carbohydrate, and one of unknown nature. The coagulation system requires in addition to the earlier-recognized components—prothrombin (thrombin), thromboplastin, fibrinogen (fibrin), and calcium—certain more recently described labile factors (20, 22, 29). Of the elements making up the two systems, it has been claimed that prothrombin and complement midpiece (C'1) are the same entity (6, 7), or at least that the same molecule is involved, with different grouping being concerned in the two reactions (21). Furthermore, the procedures used to inactivate the third and fourth components of complement has been shown to remove thrombin-forming ability as well (18). The well known fact that plasma and serum of the same blood specimen have approximately the same hemolytic complement titer does not necessarily show that coagulation and complement activity are independent phenomena,

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since the presence of prothrombin and other elements of the coagulation system may be demonstrated in serum. It does indicate, however, that even if the same or some of the same components are involved, the quantitative relationships between them vary considerably in the two reactions. So much additional information has been accumulated during the past 10 years in regard to both activities that it now seems an opportune time to reinvestigate the problem of their relationship in the light of this recent knowledge.

Thus, when a study of the effect of the vitamin content of the diet upon the complement titer of guinea pig serum was undertaken last spring in connection with another project, it seemed worthwhile to carry out a parallel investigation of the coagulative properties of the plasma and thereby obtain data on both problems simultaneously. The effect of vitamin C nutrition on the two activities will be considered in the present paper.

### Literature Review

Following a number of scattered reports on the effect of scurvy on the complement titer of human and guinea pig blood, Ecker and his co-workers (3, 5) demonstrated experimentally that a correlation existed between complement activity and the ascorbic acid level of the blood of guinea pigs. They observed that complement titers rose as the ascorbic acid content of the blood was increased to 1.0 mgm.%, but that above this level, no further increase occurred. Agnew, Spink, and Mickelsen (1, 24), on the other hand, were unable to note any relationship either *in vivo* or *in vitro*, between the ascorbic acid content of human or guinea pig blood and complement titer. Kapnick and Cope (11) recorded a series of experiments on the relationship of basal metabolic rate, complement titer, and the vitamin C intake of rabbits. After thyroidectomy, they found that the complement value of the serum fell whereas its ascorbic acid level remained normal. Conversely when hyperthyroidism was produced in the rabbits by the administration of thyroxin, the complement titer rose as the metabolic rate increased and the vitamin C content of the serum fell. Kodicek and Traub (12) tested groups of guinea pigs on diets supplemented with 0.5, 1.0, and 10 mgm. of ascorbic acid. Using the point of 50% hemolysis as the unit of comparison in their titrations, they found that, although the animals on the vitamin-C-deficient diet had lost weight, their complement titers were not altered significantly. Experiments made in the summer and late fall showed essentially similar results.

Considerable controversy has also arisen as to whether vitamin C affects the coagulability of blood. According to Macrae (14), Sir Almoth Wright described scurvy as "an acid intoxication which eventuates in a defect of blood coagulability," although Macrae himself did not note any appreciable deviation from normal in such persons. Several groups of workers subsequently reported that animals on diets low in vitamin C show an increase in plasma prothrombin time, but in general their conclusions were based on observations with small groups of animals. In a recent study, Sullivan *et al.* (25) found that scorbutic animals showed an increased response to dicumarol which became

greater the longer the deficiency lasted. Prothrombin levels took longer to return to normal after dicumarol injection in guinea pigs fed a vitamin-C-deficient diet than in animals on adequate diets. The observation of Richards and Cortell (23) that, in scorbutic guinea pigs, dicumarol caused fatty infiltration and degeneration of the liver is of interest in view of the important role of the liver in protein metabolism. Indeed, in evaluating the effects of vitamin C on either complement or coagulation, its indirect influence on protein intake due to tooth injury and attendant difficulties in eating must always be taken into consideration.

## Methods

### *Diet of Animals*

Guinea pigs were divided on the basis of weight into two groups of 36 each, both of which were fed the same basic diet of commercial rabbit pellets.\* The first group was fed fresh green grass as a source of vitamin C and other factors; the second group received a supplement of mangels that had been stored in pits during the winter. The grass collected during the hot weather in July was dry and brittle and the roots at this time had become soft and of poor quality. Thus the vitamin C content of both kinds of supplement was lower than it had been in May.

The animals were weighed periodically. Although the first group showed a greater gain in weight, the second remained in moderately good condition, with only the occasional animal showing mild symptoms of scurvy.

### *Collection of Blood*

Animals from each group were bled from the heart at periodic intervals. Part of the blood was oxalated, the remainder was allowed to clot, and the serum drawn off within two hours.

### *Coagulation Tests*

The oxalated bloods were centrifuged immediately and the plasmas tested for prothrombin activity by the methods of Quick and of Howell (13).

### *Complement Titrations*

The amount of each complement required for 50% hemolysis of a maximally sensitized sheep-red-cell suspension (the unit) was determined as described in the Standard Methods of the Division of Laboratories and Research of the New York State Department of Health (26). Complement titrations were made at approximately 2 and 24 hours after collection of the blood. A small number of sera were titrated for their content in the four major complement components, C'1, C'2, C'3, and C'4, using the method of Bier *et al.* (2).

\* *Master Rabbit Pellets from the Toronto Elevator Company, Limited, Toronto, guaranteed to contain a minimum of 15% crude protein, 4% crude fat, and a maximum of 11% crude fiber. The specific ingredients were stated to be as follows:*

*dehydrated alfalfa meal, ground yellow corn and/or hominy feed, alfalfa meal, soybean oil meal, linseed oilcake meal, feeding bone meal, ground wheat, wheat shorts, ground oats, wheat germ, wheat bran, ground limestone, 0.25% iodized salt, manganese sulphate, and fish oil fortified with vitamins A and D.*

### *Ascorbic Acid Determinations*

The method of Mindlin and Butler (19) as described for use with the Klett-Summerson photoelectric colorimeter was employed.

### **Results**

In an experiment conducted just previous to the beginning of the present study, it had been found that the majority of guinea pigs on the basic pellet diet, without roots or vitamin C supplement, would develop symptoms of scurvy and a considerable number die without showing any significant decrease in complement titer. A few animals, however, which had become greatly emaciated, had ceased to eat, and were on the point of death, showed complement titers significantly lower than those of guinea pigs on the same basic diet with roots supplement. Aside from the effect of scurvy in reducing the food intake of the animals, the omission of vitamin C from the diet for two to four weeks had no consistent reducing effect on complement activity.

It seemed possible, however, that a state of chronic vitamin C deficiency in which the metabolic disturbance extended over a longer period might be more likely to be reflected in an altered character of the blood constituents. To test the effect of chronic scurvy on complement titer in guinea pigs, two diets were selected, one to supply a large amount of vitamin C (fresh green grass supplement), the other a relatively smaller but sufficient amount to maintain the animals without any marked scorbutic symptoms (stored roots supplement). The experiment was continued from the end of April to about the middle of July.

### *Ascorbic Acid Values of Plasma*

Throughout the period of observation the second group showed very little ascorbic acid in their plasma, whereas in May and early June the first group had high values. In mid-June, after a few days of very warm weather, a decline was noted in the vitamin C level of the plasmas of the first group, a decline traceable in part at least to the decrease in the ascorbic acid content of the grass supplement. The ascorbic acid values for the May bleedings showed considerable irregularity, later found to be due to the presence of traces of copper or other substances in the distilled water diluent. These difficulties were avoided by the use of water redistilled in glass. In view of the inconsistencies in the earlier results they have not been included in Table I.

### *Complement Titers*

Table II, in which the complement titers of the various bleedings are summarized, indicates that the range and mean values for the two dietary groups were closely comparable. Both the 2- and 24-hr. complement titers have been given since it is well known that some complements, initially of very high titer, may deteriorate appreciably on standing overnight in the refrigerator. Such increased lability has been noted more particularly with complements

TABLE I

A COMPARISON OF THE ASCORBIC ACID CONTENT OF THE PLASMA OF GROUPS OF GUINEA PIGS ON DIETS OF COMMERCIAL RABBIT PELLETS SUPPLEMENTED WITH FRESH GREEN GRASS OR STORED ROOTS

Date of bleeding, 1949	Number of guinea pigs	Dietary supplement	Plasma ascorbic acid		
			Range, mgm. %	Mean, mgm. %	Standard deviation
June 1	6	Grass	1.92 - 2.83	2.29	
June 2	8	Grass	1.09 - 1.56	1.30	
June 14	7	Grass	0.32 - 1.72	1.25	
June 15	4	Grass	0.09 - 1.36	0.77	
June 20	4	Grass	0.18 - 2.00	0.74	
July 19	5	Grass	0.36 - 0.90	0.64	
July 25	7	Grass	0.36 - 0.70	0.62	
June 1	5	Roots	0.00 - 0.03	0.01	
June 2	8	Roots	0.00 - 0.18	0.04	
June 15	4	Roots	0.00 - 0.32	0.10	
June 20	8	Roots	0.00 - 0.27	0.12	
July 19	9	Roots	0.00 - 0.07	0.05	
July 25	6	Roots	0.00 - 0.10	0.04	
Mean values	41	Grass	0.09 - 2.83	1.14	0.641
" "	40	Roots	0.00 - 0.32	0.06	0.077

TABLE II

COMPLEMENT TITERS\* OF SERA OF GUINEA PIGS ON THE TWO DIFFERENT DIETS

Date of bleeding, 1949	Number bled	Supplement	First day†		Second day†	
			Range, units/ml.	Mean, units/ml.	Range, units/ml.	Mean, units/ml.
May 2	4	Grass	2000 - 2330	2130	1430 - 1820	1560
May 5	3	Grass	1790 - 2080	1960	1540 - 2380	1750
June 1 and 2	14	Grass	1370 - 2500	1790	1150 - 2000	1490
June 14 and 15	8	Grass	1430 - 2780	1890	1220 - 2000	1430
June 20	4	Grass	1920 - 3300	2500	1250 - 1430	1370
July 7	4	Grass	1300 - 1670	1490	1250 - 1820	1540
July 19	5	Grass	1590 - 2200	1720	1370 - 1540	1430
May 2	16	Roots	1560 - 2570	2270	1180 - 1540	1350
May 5	8	Roots	1430 - 2500	1990	1490 - 2280	1840
June 1 and 2	13	Roots	1430 - 2500	1790	1110 - 2220	1410
June 14 and 15	4	Roots	1320 - 2500	1850	1390 - 2330	1610
June 20	15	Roots	1320 - 3300	2330	1370 - 2270	1610
July 7	4	Roots	1250 - 2940	1850	1330 - 2000	1590
July 19	9	Roots	1540 - 2270	1790	1430 - 1820	1590
Mean values	42	Grass	1330 - 3300	1850	1150 - 2380	1490
" "	69	Roots	1250 - 3300	2000	1110 - 2330	1540

\* The titers are expressed in terms of the estimated number of 50% hemolytic units per milliliter of serum.

† Tested approximately 2 and 24 hr. after bleeding.

collected in late winter or after periods of very warm weather. No differences in relative stability were noted, however, in the bleedings from the two groups of guinea pigs.

Ten sera from each group, May bleedings, were titrated for their content in C'1, C'2, C'3, and C'4. No significant differences in mean values were recorded:

Component	Unit of comparison	Dietary supplement, units/ml.	
		Grass	Roots
C'	50% hemolysis	1527	1715
	100% hemolysis	778	926
C'1	100% hemolysis	2330	2105
C'2	100% hemolysis	723	744
C'3	100% hemolysis	1130	1367
C'4	100% hemolysis	6500	4434

### *Prothrombin Time*

The prothrombin time values of the plasmas from the same bleedings are given in Table III. The mean values for the two groups, as determined by the methods of Quick and of Howell, agreed closely, their differences being without statistical significance. In general the Quick prothrombin time values were considerably shorter than those determined by the Howell method. Certain of the June 20 bleedings, however, which were taken after the first hot spell of the season, had Howell prothrombin time values as much as 17 sec. shorter than the Quick prothrombin time values. Since the major difference in the two methods is that thromboplastin is added to the Quick but not in the Howell test, this suggested that these particular June 20 plasmas may have contained such exceptionally large amounts of thromboplastic materials that further addition was not required. Alternatively, there might have been a decrease in a thromboplastin inhibitor. The apparent effect of sudden changes in weather conditions on coagulative behavior seemed of some interest.

### *Comparison of Complement Titer, Prothrombin Time, and Ascorbic Acid Content*

Both the complement titers and the prothrombin time values for the two groups of guinea pigs ranged within what has been considered to be "normal limits". When the sera were divided into three groups on the basis of complement titers, the mean Quick and Howell prothrombin time values were found to be closely comparable for all three categories (Table IV). The higher complement titers of the 14 specimens in the first division were not associated with shorter prothrombin time values nor were the slightly lower complement

TABLE III

A COMPARISON OF THE PROTHROMBIN TIME VALUES DETERMINED BY THE METHODS OF QUICK AND HOWELL FOR PLASMA OF GUINEA PIGS ON THE TWO DIFFERENT DIETS

Date of bleeding, 1949	Number bled	Supplement	Prothrombin time					
			Quick			Howell		
			Range, sec.	Mean, sec.	Standard deviation	Range, sec.	Mean, sec.	Standard deviation
May 2	2	Grass	25 - 27	26.0		41 - 45	43.0	
May 5	3	Grass	24 - 28	25.3		24 - 34	28.6	
June 1	6	Grass	24 - 31	27.9		33 - 61	44.5	
June 2	8	Grass	22 - 31	26.6		38 - 61	47.2	
June 14*	7	Grass	28 - 44	29.7		34 - 48	41.2	
June 15*	4	Grass	24 - 31	29.4		40 - 60	54.5	
June 20*	4	Grass	23 - 39	33.3		20 - 33	26.7	
July 7*	4	Grass	25 - 37	30.1		40 - 67	47.5	
July 19	5	Grass	28 - 42	32.6		43 - 57	47.6	
July 25	7	Grass	26 - 37	31.0		35 - 50	42.1	
May 2	16	Roots	19 - 36	27.7		29 - 59	41.8	
May 5	9	Roots	29 - 41	36.2		30 - 55	43.4	
June 1	5	Roots	26 - 31	29.2		32 - 48	40.6	
June 2	8	Roots	23 - 29	27.5		37 - 45	40.2	
June 15*	4	Roots	15 - 65	34.7		32 - 69	55.0	
June 20*	8	Roots	29 - 50	35.0		21 - 49	39.8	
June 22*	8	Roots	27 - 46	33.3		33 - 46	43.6	
July 7*	4	Roots	26 - 37	31.0		37 - 61	48.7	
July 19	9	Roots	25 - 43	31.1		33 - 53	41.4	
July 25	6	Roots	31 - 39	34.6		42 - 57	49.5	
Mean values	50	Grass	22 - 44	30.0	5.29	20 - 61	42.9	9.89
" "	77	Roots	15 - 65	31.7	7.00	21 - 69	43.3	8.47

\* A period of unusually warm weather for this region.

TABLE IV  
COMPARISON OF COMPLEMENT TITERS AND PROTHROMBIN TIME VALUES

Complement titer,* units/ml.	Number of sera	Prothrombin Time			
		Quick		Howell	
		Range, sec.	Mean, sec.	Range, sec.	Mean, sec.
>2500	14	19 to 39	29.6	20 to 55	36.8
1500 - 2500	87	15 to 65	30.8	21 to 69	44.7
<1500	15	23 to 50	31.6	32 to 49	40.6

\* Minimum titer = 1250 units/ml.

Maximum titer = 3570 units/ml.

The complement titers given are those obtained approximately two hours after bleeding, that is concurrently with or shortly after the coagulation tests were performed.

titors of the 15 specimens in the third category accompanied by higher prothrombin time values. The factors responsible for these minor variations within the "normal" range were apparently different for the two activities.

In Table V the bleedings have been arranged in six groups according to their plasma - ascorbic-acid levels and independently of diet, although obviously the majority of plasmas in the lower categories were from guinea pigs in the second

TABLE V

A COMPARISON OF PLASMA ASCORBIC ACID CONTENT WITH COMPLEMENT TITERS AND WITH PROTHROMBIN TIME VALUES

Ascorbic acid values, mgm. %	Number of animals	Complement titer				Prothrombin time			
		First day		Second day		Quick		Howell	
		Range, units/ml.	Mean, units/ml.	Range, units/ml.	Mean, units/ml.	Range, sec.	Mean, sec.	Range, sec.	Mean, sec.
0 to 0.19	37	1430 to 3250	1820	1110 to 2330	1520	15 - 65	31.9	21 - 69	43.4
0.20 to 0.59	8	1670 to 3300	2000	1250 to 2000	1420	25 - 42	32.7	22 - 57	43.7
0.60 to 0.99	10	1590 to 2630	2000	1370 to 2000	1560	26 - 37	31.1	33 - 57	43.4
1.00 to 1.39	11	1350 to 3130	2080	1140 to 2220	1470	23 - 37	29.8	39 - 61	49.7
1.40 to 1.79	5	1430 to 2780	1770	1430 to 2000	1640	26 - 44	30.1	34 - 56	42.0
1.80 to 2.85	7	1430 to 2500	2040	1250 to 1670	1520	24 - 38	29.0	20 - 61	39.8

\* Titer expressed in 50% hemolytic units.

dietary group and all of those in the higher categories from the first dietary group. The range and mean complement titers and prothrombin time values have been assembled and appear closely comparable for each category.

In brief, therefore, although the ascorbic acid content of the respective plasmas had not only differed widely in the two dietary groups, but had also varied significantly within the first group, this was not reflected in any consistent change either in complement titer or in prothrombin time.

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# THE FRACTIONATION OF PHOSPHORUS CONTAINING CONSTITUENTS IN THE ALLANTOIC MEMBRANE OF THE EMBRYONATED EGG<sup>1</sup>

By A. F. GRAHAM

## Abstract

A procedure is described which permitted the phosphorus containing constituents in allantoic membranes of embryonated eggs to be separated into fractions as follows: alcohol soluble phosphorus from which the phospholipids were separated, phosphorus soluble in 5% trichloroacetic acid in which inorganic phosphorus was determined, and nucleic acid phosphorus which was further separated into pentose and desoxypentose nucleic acids. This procedure was applied to membranes between 9 and 13 days of age. It was found that the total phosphorus amounted to 9 to 10 mgm. per gm. of dried tissue. Alcohol soluble phosphorus accounted for approximately 28%, acid soluble phosphorus for 37%, and nucleic acid phosphorus for 35% of the total phosphorus. About half the acid soluble phosphorus was inorganic and about 85% of the alcohol soluble phosphorus was associated with phospholipids. These proportions remained essentially constant over the period studied. The ratio of pentose to desoxypentose nucleic acid phosphorus also remained fairly constant over the interval at about 2.2. There was no significant difference in the amounts of P<sup>32</sup> taken up over a period of 72 hr. by normal allantoic membranes and those infected with influenza virus, when inorganic radioactive phosphorus was placed in the allantoic sacs of 11-day embryonated eggs.

## Introduction

Previous work has demonstrated (3) that influenza virus A growing in the allantoic membrane of the embryonated egg in the presence of radioactive inorganic phosphorus incorporated the isotope into its structure. Both the nucleic acid and phospholipid components of the virus were found to contain the isotope (2).

In view of these findings the question arose as to whether nucleic acid and phospholipid already present in the cell were incorporated into the virus or whether these components of the virus were synthesized after infection of the cell. As an aid in answering this question, it was considered that some information should be obtained on the rate of uptake of P<sup>32</sup> by the constituents of normal cells.

Prior to this study it was necessary to determine the distribution of phosphorus among the various components of allantoic membrane. The present paper describes the techniques employed to separate the phosphorus of allantoic membrane into fractions soluble in alcohol, 5% trichloroacetic acid, and a residue containing nucleic acid and phosphoprotein. Further, the phospholipid present in the alcohol soluble fraction, inorganic phosphorus in the trichloroacetic acid soluble fraction, and the proportion of pentose to desoxypentose nucleic acid were estimated. This procedure, which, in many respects,

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is similar to that described by McCarter and Steljes (4) for rat tissue, was applied to membranes collected from fertile eggs 9 to 13 days of age and the results are described.

Some preliminary results on the uptake of P<sup>32</sup> by allantoic membranes before and after infection with influenza virus are also described.

## Methods

### *Estimation of Total Phosphorus*

The method for determining total phosphorus has been described in a previous paper (3).

### *Estimation of Radioactive Phosphorus*

A liquid Geiger-Müller counter, capacity 10 ml., as described by Veall (7) was used in conjunction with a conventional scale of 64 unit. The counter was calibrated against a standard Ra D + E source from the National Bureau of Standards, Washington, as previously described (2). In this paper the activities of radioactive sources are recorded as counts per minute (c.p.m.) 1000 c.p.m. being equivalent to 86  $\mu$ rd.

### *Preparation of Allantoic Membranes for Analysis*

Embryonated eggs from a pure bred White Leghorn flock, previously candled to ensure that they were alive, were opened at the air sac end, an incision was made in the allantoic membrane and the contents of the egg were poured out. Generally the allantoic membrane was left adhering to the inside of the egg; it was rapidly removed, rinsed twice in ice-cold 0.85% sodium chloride (w/v) immediately frozen and stored at -20° C. in a screw capped vial. Before use the material was immersed in liquid nitrogen in a stainless steel mortar, pulverized, dried *in vacuo* from the frozen state and stored over P<sub>2</sub>O<sub>5</sub> in a vacuum desiccator. No attempt was made to choose eggs of a particular weight.

## Experimental

### *Extraction of Trichloroacetic Acid Soluble Phosphorus and Alcohol Soluble Phosphorus from Membrane Tissue*

To determine whether extraction of dried membrane with 5% trichloroacetic acid (TCA) would influence later extraction of alcohol soluble phosphorus, an experiment was performed in which one quantity of tissue was extracted with boiling alcohol and then with 5% TCA and a second quantity was extracted with 5% TCA followed by extraction with hot alcohol.

Finely powdered tissue, 0.232 gm., was extracted with 5 ml. of boiling absolute alcohol (freshly purified by refluxing over solid potassium hydroxide and then distilling through a fractionating column) for five minutes in a centrifuge tube fitted with a small reflux condenser. After centrifuging the supernatant solution was removed and made to 25 ml. volume. This procedure was repeated twice more on the residue, and phosphorus estimations were carried out on each extract.

The extracted residue was allowed to dry at 37° C. and homogenized in 5 ml. of ice-cold 5% TCA for two minutes in a homogenizer of the type described by Potter and Elvehjem (5). During this procedure the homogenizer was surrounded with crushed ice. The mixture, with 2 ml. of 5% TCA used to wash out the grinder, was centrifuged and the supernatant made to 25 ml. volume. The residue was extracted twice more with 5% TCA in the same manner and phosphorus analyses were carried out on each extract.

In the second part of the experiment the above extraction sequence was reversed. The tissue, 0.232 gm., was extracted three times with 5 ml. of 5% TCA as above. The residue was resuspended in 5 ml. of absolute alcohol to remove residual TCA, centrifuged, and this was repeated. The two alcohol washes were combined and made to 25 ml. This solution is designated "1st alcohol extract". Two further extractions of the tissue with boiling absolute alcohol for five minutes each were carried out and the three extracts were analyzed for phosphorus.

The results of this experiment are shown in Tables I and II. It is seen that the amount of phosphorus extracted from the tissue by boiling alcohol or 5%

TABLE I

AMOUNTS OF PHOSPHORUS PRESENT IN SUCCESSIVE ALCOHOL EXTRACTS OF ALLANTOIC MEMBRANE BEFORE AND AFTER EXTRACTION WITH TRICHLOROACETIC ACID, 0.232 GM.  
DRY TISSUE USED IN EACH CASE

Fraction	Alcohol extracts before TCA extraction of tissue, μgm. P	Alcohol extracts after TCA extraction of tissue, μgm. P
1st extract	380	373
2nd extract	53	66
3rd extract	14	2

TABLE II

AMOUNTS OF PHOSPHORUS PRESENT IN SUCCESSIVE TRICHLOROACETIC ACID EXTRACTS OF ALLANTOIC MEMBRANE BEFORE AND AFTER EXTRACTION OF THE TISSUE WITH  
ALCOHOL, 0.232 GM. DRIED TISSUE USED IN EACH CASE

Fraction	5% TCA extracts before alcohol extraction of tissue, μgm. P	5% TCA extracts after alcohol extraction of tissue, μgm. P
1st extract	525	508
2nd extract	40	35
3rd extract	7	5

TCA was the same regardless of which solvent was employed first. Further, three extractions of the tissue with either solvent were sufficient to remove all the phosphorus compounds soluble in that solvent. This observation was

amply confirmed in other experiments. In some experiments a mixture of alcohol and ether (3/1, v/v) was used instead of absolute alcohol alone with similar results.

During extraction with TCA in the glass homogenizer a considerable amount of glass was ground off and became mixed with the tissue. Control experiments showed that this glass did not interfere in the phosphorus estimation.

*Separation of Phospholipids from the Alcohol Soluble Fraction of Allantoic Membrane*

An aliquot of the alcohol extract of tissue was evaporated to dryness at 30° C. in a stream of nitrogen. The residue was extracted three times for 10 min. each with warm petroleum ether (b.p. 40° – 60° C.) and the extracts, containing the phospholipids, were combined and analyzed for phosphorus.

To determine whether extraction of the tissue with 5% TCA prior to extraction with alcohol influenced the separation of phospholipid, the above procedure was applied to two alcohol extracts of membrane. The first alcohol extract was obtained after the tissue had been extracted with 5% TCA and the second was prepared directly from dried tissue. In both cases phosphorus analyses were carried out on the petroleum ether soluble fraction (phospholipid) and on the petroleum ether insoluble residue, the results being shown in Table III.

TABLE III

SEPARATION OF PETROLEUM ETHER SOLUBLE PHOSPHORUS FROM ALCOHOL SOLUBLE FRACTION OF DRIED ALLANTOIC MEMBRANES

Fraction	Alcohol extract prepared before TCA extraction of membranes, μgm. P	Alcohol extract prepared after TCA extraction of membranes, μgm. P
Petroleum ether soluble	34.2	20.2
Petroleum ether insoluble	8.0	22.0
Total alcohol soluble P added	45.7	44.7

It would appear that separation of phospholipid was less complete in the case where the membrane had been treated with TCA prior to alcohol extraction. It is noteworthy that this alcohol extract contained three to four times the amount of solid material found in the other alcohol extract. It was concluded that the phospholipids were more difficult to separate from the alcohol soluble material when the tissue had been subjected to previous treatment with TCA.

*Estimation of Inorganic Phosphate in Trichloroacetic Acid Extracts of Membrane*

In estimating inorganic phosphorus in trichloroacetic acid extracts of tissue the usual colorimetric method (3) was applied to the extracts without prior

acid digestion. To determine whether the sulphuric acid and molybdate present in the reagent might liberate inorganic phosphorus from easily hydrolyzable compounds in the extracts, the following experiment was performed.

A small amount of dried tissue was extracted in the cold with 5% TCA. Aliquots of the extract were added to three tubes containing molybdate and sulphuric acid, in the proportions required by the method, and the volumes made up to about 13 ml. with water. The color was developed in the first tube immediately by addition of stannous chloride solution and in the other tubes when they had stood 15 min. and 30 min. respectively at room temperature. The amounts of phosphorus estimated in the three tubes were identical.

A further experiment was carried out to find whether inorganic phosphorus might be liberated by the action of TCA during preparation of the extracts. A small quantity of tissue was extracted as rapidly as possible in the cold with 5% TCA. After centrifuging in the cold an aliquot was taken immediately from the supernatant solution for estimation of inorganic phosphorus. The remaining extract was kept at 37° C. and estimations of inorganic phosphorus were carried out at intervals over a period of 90 min. There was no change in the amount of inorganic phosphorus estimated over this period.

As a further check, the inorganic phosphorus contents of several TCA extracts of tissue were estimated using the modification of the method of Delory (1) described in a previous paper (2). Inorganic phosphorus was also estimated directly in these extracts as described above. On the average the amount of inorganic phosphate found from the direct estimation was about 10% higher than that found with Delory's method.

#### *Estimation of Pentose Nucleic Acid (PNA) and Desoxypentose Nucleic Acid (DNA) in Allantoic Membrane*

The method used was that of Schmidt and Thannhauser (6) and as applied in the present work was as follows. To tissue (0.100–0.250 gm.) which had been extracted with alcohol and 5% TCA, as described in preceding sections, was added 4.0 ml. of 1.0 *N* potassium hydroxide. The mixture was incubated 18–20 hr. at 37° C., during which time the tissue went into solution, and was then centrifuged to remove ground glass resulting from the preliminary TCA extraction in the homogenizer. Aliquots of supernatant were taken for estimation of total nucleic acid phosphorus. To 3 ml. of supernatant was added 3 ml. of 5% TCA and 0.6 ml. of 6 *N* hydrochloric acid. The precipitated DNA and protein was centrifuged off and washed once with 2.5 ml. of 5% TCA. This wash was added to the first supernatant, the total volume was made to 10 ml. and phosphorus analyses were carried out to determine PNAP. About 1 ml. of 0.1 *N* potassium hydroxide was added to the DNA precipitate which was dissolved by warming, the volume was made up to 10 ml., and the DNAP content was estimated by phosphorus analysis.

To determine the efficiency of the method in separating DNA and PNA, two samples of lipid free, TCA extracted tissue of equal weight were taken.

To one of these were added known amounts of calf thymus nucleic acid and yeast nucleic acid. Both samples were submitted to the separation procedure described above. The results are shown in Table IV.

TABLE IV

SEPARATION OF YEAST NUCLEIC ACID AND THYMUS NUCLEIC ACID, ADDED TO LIPID FREE, TRICHLOROACETIC ACID EXTRACTED ALLANTOIC MEMBRANE TISSUE, BY THE METHOD OF SCHMIDT AND THANNAUSER

	Total phosphorus, μgm.	PNA phosphorus, μgm.	DNA phosphorus, μgm.
Membrane (0.118 gm.) with added PNA and DNA	468	297	168
Membrane (0.118 gm.) alone	324	230	92
Recovered	144 (94%)	67 (93%)	76 (94%)
Added	153	72	81

It is seen that the recovery of added PNAP and DNAP was 93 to 94% of that added.

On several occasions DNAP and PNAP, after separation by the Schmidt and Thannhauser method, were estimated by the diphenylamine and orcinol color reactions as previously described (2). The diphenylamine reaction was applied after the DNA had been extracted from the protein with 5% TCA for 45 min. at 95° C. This extraction removed all but 3% of the phosphorus of the DNA fraction.

The results obtained with these color tests varied a good deal, the orcinol reaction indicating 15 to 35% more PNAP than direct phosphorus estimation and the DNAP being 12 to 25% higher by the diphenylamine reaction than by phosphorus analysis.

#### *Distribution of Phosphorus Constituents in Allantoic Membranes*

As a result of the above experiments the following procedure was adopted to fractionate the phosphorus containing constituents of allantoic membranes.

The dry weighed material, 100 to 350 mgm., was extracted with boiling absolute ethanol, or ethanol-ether (3/1, v/v), phosphorus analyses being carried out on the extract. An aliquot of the alcohol extract was evaporated to dryness and thoroughly extracted with petroleum ether, to remove phospholipids, and this solution was analyzed for phosphorus. The alcohol insoluble material was homogenized with 5% TCA, total and inorganic phosphorus being estimated in the extract. The Schmidt and Thannhauser technique was applied to the residue to separate the nucleic acids. PNAP as estimated here includes the phosphorus from any phosphoprotein which may have been present in the membrane, no attempt being made to estimate the phosphoprotein separately.

This analytical procedure was applied to membranes collected at 24 hourly intervals from embryonated eggs 9 to 13 days of age. Seven or eight membranes were collected at each time, pooled, and analyzed together. The results are shown in Figs. 1, 2, and 3. In Fig. 1 the phosphorus contents of the various

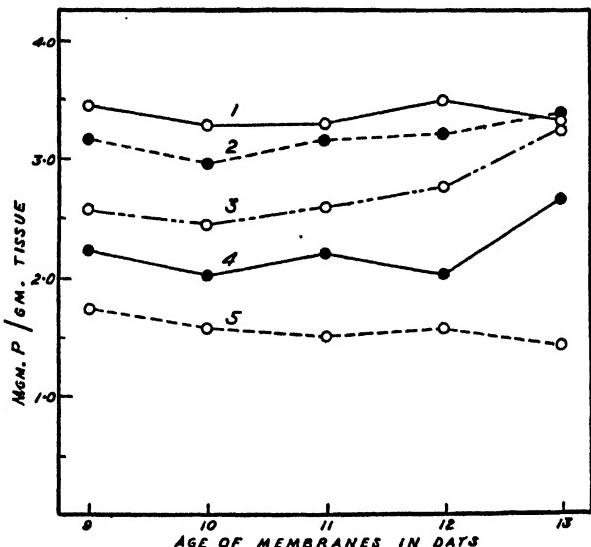


FIG. 1. Phosphorus contents in mgm. per gm. of dry tissue of the various fractions of allantoic membranes between 9 and 13 days of age. Curve 1, 5% trichloroacetic acid soluble phosphorus. Curve 2, total nucleic acid soluble phosphorus. Curve 3, alcohol soluble phosphorus. Curve 4, petroleum ether soluble phosphorus (phospholipid). Curve 5, inorganic phosphorus, present in 5% trichloroacetic acid soluble fraction.

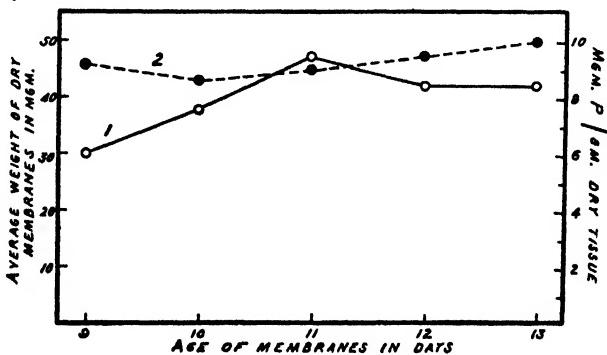


FIG. 2. Relationship of average dry weight of allantoic membranes, Curve 1, and total phosphorus content, Curve 2, to age of membranes.

fractions calculated in terms of mgm. P per gm. dry tissue are plotted against the age of the membrane in days. In Fig. 2 are plotted the average weights of the membranes in mgm. and the total phosphorus content of the membranes as mgm. per gm. dry tissue. The figures for the total phosphorus contents of the membranes were obtained by addition of the results from the individual

fractions shown in Fig. 1. Fig. 3 shows the ratio of PNAP (including phosphoprotein phosphorus) to DNAP, obtained in three separate experiments, plotted against age of membrane.

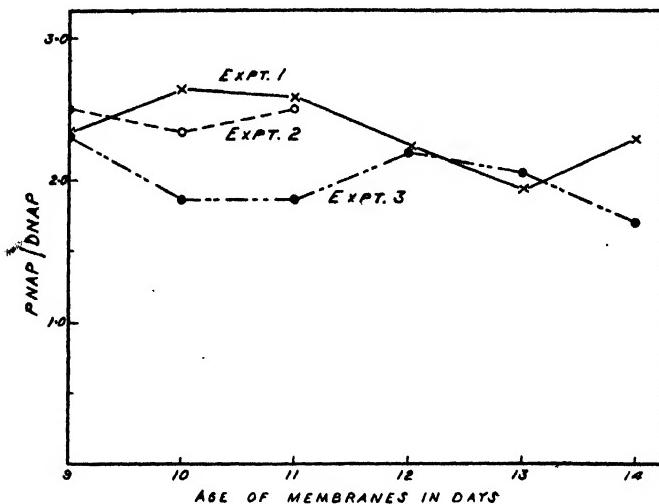


FIG. 3. *The ratio of pentose nucleic acid phosphorus, PNAP, to desoxypentose nucleic acid phosphorus, DNAP, in allantoic membranes between 9 and 14 days of age.*

The results shown in Figs. 1 and 2 for a single experiment were similar to those obtained in a second complete experiment carried out on membranes 9 to 14 days of age.

#### *Uptake of Radioactive Phosphorus by Normal Allantoic Membranes and Membranes Infected with Influenza Virus A*

The general procedure for determining the uptake of  $P^{32}$  by allantoic membranes was as follows.  $P^{32}$ , as inorganic phosphate in 0.85% sodium chloride solution, was injected from a calibrated syringe into the allantoic fluid of 25 to 30 11-day embryonated eggs, 0.2 ml. containing 3000 to 4000 c.p.m. into each egg. The eggs were then incubated at 36° C. and at intervals the membranes from a group of five or six eggs were collected. After being washed thoroughly in two changes of ice-cold saline, the membranes were digested in a mixture of 5 ml. of concentrated sulphuric acid and 2 ml. of 72% perchloric acid and the volume was made up to 50 ml. Radioactivity estimations were carried out on these solutions.

In one experiment with 11-day-old embryonated eggs the uptake of  $P^{32}$  by normal membranes was determined over a period of 115 hr. and the results are shown in Fig. 4 as Curve 3. A second experiment was carried out in which two groups of 25 11-day eggs were chosen at random from a large number. Influenza virus A (PR8 strain) was injected in 0.2 ml. amount (10000 ID<sub>50</sub>) by the allantoic route into each egg of the first group.  $P^{32}$  was then injected

into all the eggs which were incubated at 36° C. The  $P^{32}$  contents of the membranes of each group were determined at intervals as described above and the results are plotted in Fig. 4, as Curves 1 and 2.

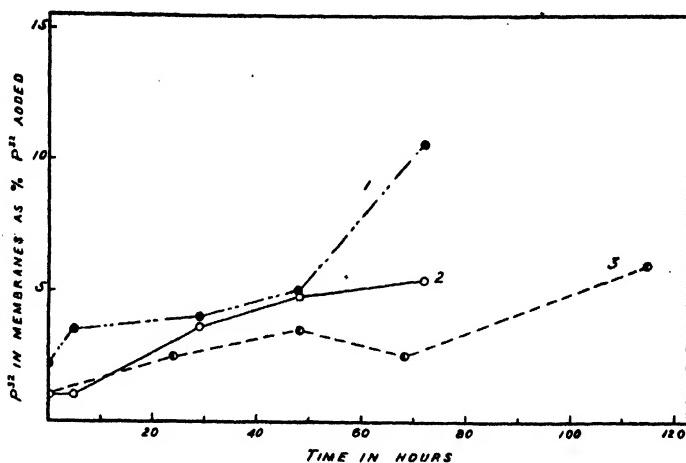


FIG. 4. Uptake of  $P^{32}$  by allantoic membranes from inorganic radioactive phosphorus placed in the allantoic sacs of 11-day embryonated eggs. Curve 1 represents uptake of  $P^{32}$  for eggs infected with influenza virus and Curve 2 for normal embryonated eggs, the experiments being carried out simultaneously. Curve 3 represents uptake of  $P^{32}$  by normal membranes in a separate experiment.

### Discussion

The procedure described permitted the phosphorus constituents of allantoic membrane to be separated into several fractions, acid soluble phosphorus in which the inorganic phosphorus was determined, alcohol soluble phosphorus from which the phospholipids were separated, and nucleic acid phosphorus which was further divided into PNAP and DNAP.

When this procedure was applied to membranes between 9 and 13 days of age the amounts of acid soluble, inorganic, alcohol soluble, phospholipid, nucleic acid, and total phosphorus per gram of tissue remained essentially constant over this interval. Acid soluble P accounted for about 37%, alcohol soluble P for about 28%, and nucleic acid P for about 35% of the total phosphorus of the membrane. In a second such experiment the figures were 46%, 21%, and 33% respectively. The total phosphorus in the membranes amounted to 9 to 10 mgm. per gm. of dried tissue. About half the TCA soluble P was inorganic and about 84% of the alcohol soluble P was contained in phospholipids. Since the membranes on the average increased about 80% in weight between 9 and 11 days the actual amounts of the various phosphorus constituents increased by roughly the same proportion.

Although there was considerable variation in the PNAP/DNAP ratio this ratio may be greatly influenced by small errors in analysis and it is considered that it remains essentially constant at about 2.2 over the 9 to 14 day period.

It is doubtful whether there was any significant difference between the amounts of  $P^{32}$  taken up by normal and virus infected 11-day membranes. However, further work may show that there is a significant difference in the rates with which the various components of the membrane are labelled in the two cases. It would be of interest to determine whether the rate of uptake is faster by nine-day membranes than by those 11 days of age since the membrane weight and phosphorus content increase roughly twofold between 9 and 11 days. It seems possible that the  $P^{32}$  in the allantoic fluid is not readily utilized by the membrane cells. If this is so, it would be worthwhile to determine the main source of the phosphorus utilized by these cells. A method might then be devised for increasing the specific radioactivity of influenza virus grown in the membrane.

#### Acknowledgment

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# STUDIES ON THE RELATIONSHIP BETWEEN VIRUS AND HOST CELL: THE PREPARATION OF T2<sup>r+</sup> BACTERIOPHAGE LABELLED WITH RADIOACTIVE PHOSPHORUS<sup>1</sup>

By S. M. LESLEY, R. C. FRENCH, AND A. F. GRAHAM

## Abstract

T2<sup>r+</sup> bacteriophage grown in its host, *Escherichia coli* B, in broth medium in the presence of radioactive inorganic phosphorus was labelled with the isotope. Purified suspensions of this virus had specific activities up to 50,000 c.p.m. per µgm. P. There was little or no exchange of P<sup>32</sup> between virus and inorganic phosphate. Chemical analysis showed that at least 98% of the virus phosphorus was contained in nucleic acid; of the nucleic acid phosphorus 95.5% was associated with desoxypentose nucleic acid and 4.5% with pentose nucleic acid. More than 99% of the radioactivity of the labelled bacteriophage was contained in the nucleic acid fraction. Preparations of bacteriophage were obtained with sufficiently high specific activity to enable metabolism experiments to be carried out on the growth of the labelled virus in the host cell.

## Introduction

In a previous paper (6) a method was described which enabled influenza virus growing in the embryonated egg to be labelled with radioactive phosphorus. As was pointed out the main purpose of that work was to obtain highly radioactive specimens of influenza virus to enable the metabolism of the isotope to be studied when cells were infected with the labelled virus. While little difficulty was experienced in labelling influenza virus with relatively small amounts of P<sup>32</sup>, the specific activity was not sufficiently high to enable the metabolism experiments to be carried out.

It was considered, however, that such a study might be more feasible with a different virus - host cell system, namely T2 bacteriophage active on *Escherichia coli*, strain B. It has already been demonstrated by Cohen (1) that the T2 and T4 bacteriophage of *E. coli*, and by Kozloff and Putnam (10) that the T6 bacteriophage were labelled with P<sup>32</sup> when inorganic radioactive phosphorus was added to the medium supporting virus growth in the infected cells.

The present paper describes the methods used to obtain purified highly radioactive preparations of T2<sup>r+</sup> bacteriophage labelled with P<sup>32</sup>. Experiments are described which support the conclusion that the label is contained almost entirely in the nucleic acid fraction of the virus.

Utilizing these methods preparations of bacteriophage were obtained sufficiently high in specific radioactivity to enable metabolism experiments to be carried out with the virus on its host, *E. coli*, strain B.

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## Methods and Materials

### Determination of Total Phosphorus

The method of estimating total phosphorus has been previously described in detail (6).

### Determination of Radioactive Phosphorus

The radioactivity of  $P^{32}$  solutions was estimated with a liquid Geiger-Müller counter, capacity 10 ml., of the type described by Veall (13). The counter was standardized against a RaD+E source from the National Bureau of Standards, Washington, as previously described (5). The results of radioactivity measurements, given in this paper in terms of counts per minute (c.p.m.), may be converted to disintegrations per second using the factor 1000 c.p.m. are equivalent to 86  $\mu$ rd.

Specific activity is defined as c.p.m. per  $\mu$ gm. P unless otherwise indicated.

### Preparation of Media

The liquid medium used throughout this work was a nutrient solution prepared by dissolving 20 gm. Difco Bacto-Tryptose, 5 gm. sodium chloride, and 1 gm. glucose per liter in distilled water. After adjusting the pH to about 7.2, the solution was autoclaved. Solid medium was prepared by adding agar to the broth medium before autoclaving.

### Type of Bacteriophage and Bacteria

The bacteriophage used throughout was the T2 $r^+$  strain, active on *E. coli* B, received from Dr. Fred Heagy, University of Western Ontario. Using the method of Delbrück and Luria (3) this bacteriophage was found to have a latent period of 23 min. and a burst size of 115 in Bacto-Tryptose broth.

### Determination of Bacterial Concentration in Liquid Cultures

The concentration of cells in broth culture was estimated on a Coleman Spectrophotometer at 630 m $\mu$ , the per cent transmittance being read against broth as a blank. The instrument was standardized against cell suspensions in which the number of cells was determined by colony count and also by direct count in a Petroff-Hausser counting chamber.

### Determination of Virus Concentration

The number of infective particles in a bacteriophage suspension was determined by the method of Hershey *et al.* (7). Since slight modifications were made to the original method, the procedure is described below.

Serial 10- or 100-fold dilutions of the virus solution were made in 0.85% (w/v) sodium chloride (saline) until the number of infective centers in the final dilution was between 60 and 1200. To 1 ml. of this dilution was added 3 ml. of broth suspension of *E. coli* containing about  $2 \times 10^8$  cells per ml., freshly prepared from a 15-20 hr. agar slope. One ml. aliquots of this mixture were then added to each of three tubes containing 1 ml. of melted nutrient agar (0.7%). The contents of each tube were mixed and poured over the surface

of a nutrient agar (1%) plate. When the agar had solidified the plates were incubated for 18 hr. at 37° C. and the plaques were counted. From the results the number of plaque forming particles per ml. of the original suspension was calculated and in this paper is expressed as phage per ml. The plating efficiency determined by the method of Ellis and Delbrück (4) was about 0.65.

When titrating saline suspensions of bacteriophage, prepared as described in a later section, a 100-fold dilution of the suspension was made in broth and allowed to stand at least one hour before further dilutions were made in saline. It was found that higher titers were obtained by this procedure than when the initial dilution was made in saline.

## Experimental

### *Growth and Purification of Bacteriophage*

Cultures of *E. coli* B were prepared by inoculation of 150 ml. of broth from an 18 hr. slope and grown to  $2 \times 10^8$  cells per ml. with rapid aeration at 37° C. The generation time of *E. coli* under these conditions was about 21 min. After centrifuging, the cells were resuspended in 150 ml. of fresh broth at 37° C. and immediately inoculated with T2r<sup>+</sup> bacteriophage from a stock broth culture usually in the ratio of three to five virus particles per cell. In some cases, for example in preparing phage labelled with P<sup>32</sup> as described later, the centrifuging and resuspension of the cells were omitted and the virus inoculum was added to the culture as soon as it has reached the required concentration of cells.

The culture was rapidly aerated at 37° C. until lysis occurred when the titer of phage reached  $4 \times 10^{10}$  to  $10^{11}$  phage per ml. Bacterial debris was then removed by centrifuging at 4300 g. for 30 min. in a Sorvall angle centrifuge at 5° C. About 25% of the virus was lost in this step, but further purification depended upon efficient removal of the debris. The virus was then sedimented by centrifuging the supernatant solution at 22,000 g. for one hour in the angle centrifuge. The supernatant was aspirated off until the level of liquid was just above the virus pellet which adhered to the side of the tube. When the tube was left for some time a bluish material was observed to separate slowly from the pellet and fall to the bottom of the tube. By this means a separation was obtained between virus and the nonviral material which remained adhering to the tube as an opaque tightly packed pellet. When the separation was complete, after 20–40 min., as much as possible of the remaining liquid and the pellet were carefully drawn off through a fine tipped pipette leaving the virus in the bottom of the tube as a small opalescent pool. A few ml. of 0.85% saline were then added and the virus was dispersed by giving the tube a quick swirl. The volume was made up with saline to about one-quarter the original volume, the virus was sedimented at 22,000 g. and separated from the pellet as before. The nonviral pellet was very small at this stage and was absent when the procedure was repeated a third time. The virus was finally resuspended in 10 ml. of saline, the recovery being 25 to 40% of that

present in the original lysate. With this procedure the customary intermediate low speed centrifugations to remove aggregated material after resuspension of the virus were unnecessary.

#### *Properties of the Purified Virus*

Preparations of purified virus in 0.85% saline containing  $10^{11}$  to  $4 \times 10^{11}$  phage per ml. gave single boundaries in the ultracentrifuge (Fig. 1) with an average sedimentation constant of 1040 S in agreement with the results of Sharp *et al.* (11).

Chromium shadowed preparations in the electron microscope showed the usual tadpole-shaped particles (8), the dimensions of the head being approximately 90 by 125 m $\mu$ , the length of the tail about 110 m $\mu$ .\*

Phosphorus analyses carried out on six preparations of purified phage gave 2.76, 2.67, 4.15, 2.94, 2.15,  $3.46 \times 10^{-11}$   $\mu$ gm. P per phage or an average of  $3.02 \times 10^{-11}$   $\mu$ gm. P per phage.

Saline suspensions of purified bacteriophage, kept at 5° C., did not decrease in titer over a period of eight weeks.

#### *Preparation of Bacteriophage Labelled with Radioactive Phosphorus*

In order to obtain virus of high specific activity the phosphorus content of the broth medium was decreased as follows. Twenty gm. of Bacto-Tryptose was dissolved in about 200 ml. of distilled water, the solution was adjusted to pH 8.4 and 2 ml. saturated calcium chloride solution was added. The precipitate of calcium phosphate was removed by centrifugation, sodium chloride and glucose were added in the required proportions to the supernatant solution, the volume was made to 1000 ml., and the solution autoclaved. By this means the total phosphorus content of the final medium was reduced from 140 to about 30  $\mu$ gm. P per ml.

To 150 ml. of this medium was added up to 1.5 mc. of  $P^{32}$  as inorganic phosphate, carrier free. The medium was inoculated with *E. coli* from an 18 hr. slope and grown to  $2 \times 10^8$  cells per ml. at 37° C. with aeration. Bacteriophage from a stock culture was then added to give a final concentration of 6 to  $8 \times 10^8$  phage per ml., and the suspension was aerated until lysis occurred.

The bacteriophage, which reached a final titer of  $4 \times 10^{10}$  to  $10^{11}$  phage per ml. in the lysate, was purified in the manner previously described. The virus was sedimented repeatedly, usually four to six times, until the specific activity of the supernatant solution, c.p.m. per phage, reached that of the resuspended virus. After each sedimentation the phage was resuspended in about 40 ml. of saline the final suspension being made in 8 to 12 ml. of saline. By this means suspensions of purified virus were obtained having titers of 1 to  $2 \times 10^{11}$  phage per ml., and specific activities varying from 500 to 50,000 c.p.m. P depending on the amount of  $P^{32}$  added to the medium.

\* We are indebted to Dr. G. D. Scott of the Physics Department for the electron micrographs.

PLATE I



FIG. 1. Sedimentation diagrams of purified T2r<sup>+</sup> bacteriophage in 0.85% sodium chloride at pH 6.05. Concentration was 1.76 mgm. phage per ml. Direction of sedimentation is from left to right. Photographs were taken at two-minute intervals with a mean centrifugal field of 11,100 g. using a Spinco Ultracentrifuge equipped with a Philpot-Svensson type of optical system.



Since these radioactive preparations were required for other experiments phosphorus analyses were carried out on only a small number. However, there appeared to be no difference in the  $\mu\text{gm. P}$  per phage between normal and radioactive preparations. Further, repeated phage titrations carried out at intervals over a period of several weeks with several highly radioactive phage preparations showed no inactivation of virus by the radiation.

*Control Experiments to Determine Whether Bacteriophage Takes up  $\text{P}^{32}$  by Exchange with Inorganic Phosphate*

To determine whether  $\text{P}^{32}$  was taken up by bacteriophage by exchange or adsorption on its surface the following experiment was carried out.

To two tubes, each containing 150 ml. of broth culture of *E. coli*, in the exponential phase of growth, at a concentration of  $5 \times 10^7$  cells per ml., was added sufficient T2 $r^+$  bacteriophage to give a ratio of approximately 1 virus particle per 40 cells.  $\text{P}^{32}$  was added immediately to tube A to give a final concentration of 75,000 c.p.m. per ml. Both cultures were then aerated at 37° C. until lysis occurred. An identical amount of  $\text{P}^{32}$  was then added to tube B as had been added to tube A and both cultures were allowed to stand overnight at 5° C. The concentration of virus in both cultures at lysis was about  $7.5 \times 10^{10}$  phage per ml. The phage was then purified as previously described and total phosphorus and radioactivity estimations were carried out on the final virus suspensions. Purified phage from tube A had a specific activity of 481 c.p.m. per  $\mu\text{gm. P}$ , while that from tube B had 1.6 c.p.m. per  $\mu\text{gm. P}$ . It was thus apparent that by far the greater proportion of  $\text{P}^{32}$  contained in radioactive phage was incorporated during growth of the virus in the host cells.

In a further experiment 1.0 ml. of  $\text{P}^{32}$  (carrier free) solution in saline was added to 1.0 ml. of purified phage to give a final  $\text{P}^{32}$  concentration of 326,000 c.p.m. per ml. and a specific activity of 28,200 c.p.m. per  $\mu\text{gm. P}$ . After standing for 18 hr. at 5° C. the virus was alternately sedimented at 22,000 g. and resuspended in 40 ml. of saline. After the fifth sedimentation the specific activity was found to be 209 c.p.m. per  $\mu\text{gm. P}$ , and after the sixth 82 c.p.m. per  $\mu\text{gm. P}$ . The final specific activity of the virus was thus 0.3% of the specific activity contained in the initial suspension, indicating little or no exchange or adsorption of radioactive inorganic phosphorus by the virus.

*Further Tests to Determine Whether  $\text{P}^{32}$  was Incorporated into the Structure of Radioactive Bacteriophage*

It was thought that if a significant proportion of the  $\text{P}^{32}$  contained in radioactive bacteriophage were adsorbed on the surface it might be released when the phage was adsorbed on the host cells. To investigate this point purified radioactive phage in broth was added to a broth suspension of *E. coli* to give a ratio of one virus particle to two cells with the final concentration of cells being about  $10^{10}$  per ml. The mixture was kept at 37° C. for seven minutes then chilled to 5° C. and centrifuged to remove the cells and adsorbed bacteriophage. It was found that the supernatant solution thereby obtained contained

14% of the added phage and 16% of the added P<sup>32</sup>. For all radioactive phage preparations so far tested by this method similar results have been obtained. The isotope would appear to be firmly fixed to the virus particle.

Since trichloroacetic acid is widely used as a solvent to extract inorganic and low molecular weight phosphorus compounds from cells it was of interest to determine whether purified radioactive phage contained phosphorus compounds soluble in trichloroacetic acid. Accordingly, a quantity of purified radioactive phage was added to 20 ml. of broth and allowed to stand an hour or so to ensure that the virus was dispersed. The solution was chilled in an ice bath and 0.2 ml. of dialyzed 1% egg albumin solution was added to act as a protein carrier, followed by 2.9 ml. of 40% trichloroacetic acid. After standing for 15 min. in the cold the precipitate was removed by centrifuging and the P<sup>32</sup> content of the supernatant determined.

This procedure was carried out on each of nine preparations of purified radioactive phage. The amount of radioactivity remaining in the trichloroacetic acid supernatant was generally less than 0.5% of that added originally with the bacteriophage. In the case of one preparation 0.9% of the added radioactivity was soluble in the trichloroacetic acid. In this particular case a suspension of the purified phage in broth was dialyzed for several days against several changes of 0.1 M phosphate buffer pH 7.0. The dialyzate was found to contain 0.9% of the radioactivity present in the bacteriophage.

It is evident from these results that the preparations of purified bacteriophage contained little P<sup>32</sup> soluble in trichloroacetic acid suggesting that the isotope was incorporated in the structure of the virus.

#### *Chemical Analysis of Radioactive Bacteriophage*

In order to obtain additional evidence that the bacteriophage was labelled, a chemical analysis was carried out on a purified radioactive specimen as follows: A preparation of purified radioactive bacteriophage suspended in saline was diluted with about eight times its quantity of purified but not radioactive phage. This gave a suspension containing sufficient virus (about 4.5 mgm.) for analysis and containing an amount of activity convenient for measurement. The preparation contained  $2.7 \times 10^{-11}$   $\mu\text{gm. P}$  per phage and a specific activity of 657 c.p.m. per  $\mu\text{gm. P}$ . To the suspension was added 0.25 ml. of 1% egg crystalline albumin to act as a protein carrier. An analytical control, which was treated throughout in the same manner as the virus, contained 0.25 ml. of the albumin solution in saline.

Sufficient 40% trichloroacetic acid was added to give a final concentration of 5% and the precipitate was removed by centrifugation. The residue was washed twice with cold absolute alcohol and then extracted twice with boiling alcohol-ether (3/1, v/v) for 15 min. The residue was then extracted three times at 95° C. with 5% trichloroacetic acid for 15 min. to remove the nucleic

acid.  $P^{31}$  and  $P^{32}$  estimations were carried out on the 5% trichloroacetic acid, alcohol-ether, and nucleic acid fractions and the nucleic acid free residue; the results are shown in Table I.

TABLE I  
CHEMICAL ANALYSIS OF RADIOACTIVE BACTERIOPHAGE

Fractions	Total $P^{31}$ , $\mu\text{gm.}$	Total $P^{32}$ , c.p.m.	Specific activity, c.p.m. per $\mu\text{gm. P}$
Whole virus	189	124000	657
5% Trichloroacetic acid soluble	4.2	1175	280
Alcohol-ether soluble	0	50	—
Nucleic acid	210	137000	653
Nucleic acid free residue	0	346	—

It is observed that the phosphorus of the virus is contained almost entirely in the nucleic acid fraction in accordance with the observations of Taylor (12). Further, practically all the radioactivity is associated with the nucleic acid.

Since Taylor (12) has reported the presence of 6.6% pentose nucleic acid and 40.3% desoxypentose nucleic acid in this virus, the orcinol reaction for pentose and the diphenylamine reaction for desoxypentose were applied to the nucleic acid fraction of the virus in the manner described in a previous paper (5). The results of these tests showed the presence of 9.8  $\mu\text{gm.}$  of pentose nucleic acid phosphorus and 204  $\mu\text{gm.}$  of desoxypentose nucleic acid phosphorus, compared to 210  $\mu\text{gm. P}$  in the fraction (Table I) estimated by phosphorus analysis.

### Discussion

From sedimentation and electron microscope studies it is concluded that the preparations of purified T2r<sup>+</sup> bacteriophage obtained in this work consisted almost entirely of the virus particles. This conclusion is supported by the finding that one plaque forming unit in the preparations contained about  $3.0 \times 10^{-11} \mu\text{gm. P}$ , a result not inconsistent with the figure of  $4.7 \times 10^{-11} \mu\text{gm. P}$  per phage calculated from the results of Hook *et al.* (8) for this virus and  $3.9 \times 10^{-11} \mu\text{gm. P}$  per phage found by Kozloff and Putnam (9) for the closely related T6r<sup>+</sup> bacteriophage of *E. coli*.

At least 98% of the phosphorus of the virus was contained in the nucleic acid fraction, the remaining 2% being soluble in 5% trichloroacetic acid. About 95.5% of the nucleic acid phosphorus was found in desoxypentose nucleic acid and about 4.5% in pentose nucleic acid. While these results confirm the observation of Taylor (12) that both nucleic acids are present in purified preparations of the virus yet recent work by Cohen (2) indicates that the PNAP found may be present as an impurity. The amount of pentose nucleic acid phosphorus determined by Taylor for this virus by a different method, namely 12.7% of the total nucleic acid phosphorus, was somewhat

higher than obtained in the present work. Kozloff and Putnam (9) have found 2.4 to 3.4% of the total phosphorus in synthetic medium T6 $r^+$  bacteriophage to be present in the nucleic acid.

Sufficient P<sup>32</sup> was introduced into the virus during its growth to enable metabolism experiments to be carried out with the labelled phage growing on the host cells. That the isotope was, in fact, incorporated into the structure of the virus was indicated by control experiments which showed little or no exchange of P<sup>32</sup> between inorganic phosphate and virus. Also chemical analysis demonstrated that at least 99% of the P<sup>32</sup> in the labelled virus was associated with the nucleic acid fraction. Within experimental error the specific radioactivity of the nucleic acid fraction was the same as that of the whole virus.

A small amount of the radioactivity of the virus was soluble in 5% trichloroacetic acid. The proportion varied from one preparation of virus to another up to about 1% but was usually less than 0.5%. It is considered, however, that this was radioactive impurity carried through the purification procedure rather than an integral part of the virus.

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# ON THE ANTIHAEMOLYTIC VALUE OF THE BLOOD OF RABBITS AS MEASURED BY LYSOLECITHIN<sup>1</sup>

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## Abstract

The antihaemolytic values (AHV) of the erythrocytes and plasma of experimental rabbits have been determined by the lysolecithin method. Oral administration of cholesterol resulted in hypercholesterolaemia; and the increase in plasma AHV paralleled the rise in free cholesterol in approximately a 1 : 1 molar ratio of lysolecithin to cholesterol. No changes in the erythrocytes were observed, other than a mild anaemia. Splenectomy followed by cholesterol feeding afforded essentially the same results. In the normal rabbit it is concluded that the free cholesterol contributes very little to the antihaemolytic value of the plasma. Repeated bleeding caused a hypercholesterolaemia, and an elevation of the plasma AHV which paralleled the free cholesterol. In the haemolytic anaemia caused by acetylphenylhydrazine injections, the chief findings were an increase in hypotonic fragility of the erythrocytes, and a decrease in the plasma AHV. In all the experiments the mean erythrocyte AHV varied with the size of the cells, but appeared to be otherwise unaffected by the experimental procedures.

## Introduction

Collier and Wilbur (10), in attempting to estimate the lysolecithin content of blood, described a method for measuring the amount of added lysolecithin (LL) which was required to produce 50% haemolysis of 1 ml. of blood under standard conditions. This amount, in mgm. of lysolecithin per ml. of blood, was termed the Antihaemolytic Value (AHV). It was assumed that there is normally an equilibrium between haemolytic and antihaemolytic factors in the circulating blood (8, 25) and that an alteration in this balance would be reflected in a change in the AHV. The chief haemolytic factors in the plasma are presumably fatty acids, bile salts, and lysolecithin; while the antihaemolytic substances are believed to be free cholesterol and the plasma proteins.

Other workers have attempted to estimate the antihaemolytic value of the blood by titration with lysins. Port (27) in 1910 used saponin for this purpose and found significant alterations in the serum of patients. Clark and Evans (5, 39) used saponin and also sodium oleate. Valette (35) measured the antihaemolytic value of serum to soaps, bile salts, and saponin; while de Vries (12) estimated the haemolytic activity of serum extracts against human erythrocytes.

May (21) in 1914 used saponin for determining the resistance of erythrocytes in disease. Singer (32) used lysolecithin for this purpose, and a haemolysis method for estimating the lysolecithin content of the serum of his patients. Foy and Kondi (16) and Maizels (20) also employed lysolecithin for determining the resistance of erythrocytes to haemolysis.

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In the present investigation lysolecithin was used for the measurement of the AHV of rabbit erythrocytes\* and plasma. The effects of splenectomy, of cholesterol feeding, and of acetylphenylhydrazine injection were observed.

### Methods

All blood specimens were taken by heart puncture and heparinized, unless otherwise stated. Haemoglobin concentration was determined by the cyanmethaemoglobin method of Collier (6). The erythrocyte counts were performed upon dilutions in Gower's fluid; 10 squares in two fields were always counted, and the standard error of the count was found to be about 0.1 - 0.2 millions per  $\mu\text{l}$ . Packed erythrocyte volume ("haematocrit") was estimated in Wintrobe tubes centrifuged for 30 min. at 3000 r.p.m. in an International Clinical Centrifuge.

The free and ester cholesterol of plasma were separated by the digitonin procedure of Sobel and Mayer (33), and the estimations were made by the Rose, Schattner, and Exton (30) modification of the Tschugaeff reaction. As this affords a much more intense color than the Liebermann-Burchard reaction it is eminently suitable for estimating the low cholesterol levels of rabbit plasma.

A turbidimetric method for plasma protein, using sulphosalicylic acid, failed to give consistent results, and all plasma protein determinations were made by a micro-Kjeldahl procedure.

Blood AHV determinations were made by a modification of the method of Collier and Wilbur (10). In the original method the time for 50% haemolysis was specified as 60 sec. but it was subsequently found (7) that the action of the lysin required a much longer time to reach completion. For this reason certain preliminary measurements (9) are of little value. The AHV is now defined as: the number of mgm. of LL required for 50% haemolysis of 1 ml. of blood (or its equivalent) in 15 min. at room temperature.

The purity of the lysolecithin preparations was estimated by a determination of lipid phosphorus, and the figure of 6.08% P was taken as representative of what is actually a mixture of lysolecithin and lysocephalin.

The AHV of whole blood was determined as follows: Six small tubes were set up, each containing 5 ml. of 1 : 125 blood in buffered saline (10). Increasing amounts of LL (1 : 1000 in absolute ethanol) were added, the contents of the tubes were mixed, and after 15 min. the degree of haemolysis was estimated photometrically as previously described (10). The percentage haemolysis was plotted against the amount of LL added, and the amount required for 50% lysis was obtained by interpolation. The weight of LL, in mgm., is multiplied by 50 to obtain the AHV of 1 ml. of blood.

\* It has been suggested that the term "Antihæmolytic Value" may not appropriately be applied to the erythrocytes. We agree with this contention, but retain the term in this sense, because of its use in previous publications. The AHV of erythrocytes could more properly be described as the 'resistance' to haemolysis by lysolecithin.

The AHV of erythrocytes, after threefold washing in buffered saline, was determined in similar fashion, the results being expressed as mgm. of LL required for the erythrocytes from 1 ml. of whole blood. The mean corpuscular antihaemolytic value (MCAHV) was calculated by dividing the erythrocyte AHV by the number of cells per ml. of blood, and is expressed as  $\mu\text{ugm}$ . of LL per cell. The MCAHV represents, of course, only 50% of the mean LL required to lyse a single cell in the blood specimen.

The AHV of plasma was determined by adding a known volume of plasma to erythrocytes of known AHV and determining the AHV of the mixture. From the difference, the AHV of 1 ml. of plasma was calculated, and this represents essentially the amount of LL which can be neutralized by the plasma.

The hypotonic fragility, or osmotic resistance, of erythrocytes was estimated by the method of Parpart *et al.* (24). The blood was diluted 1 : 125 in buffered saline of varying tonicities, and the solutions were restored to isotonicity by addition of the complementary solutions before the opacity was read in the photometer. The results were then plotted on probability paper, as suggested by Bonét-Maury and Chouteau (3) and the fraction of isotonicity corresponding to 50% haemolysis was noted. This is designated as the Mean Corpuscular Fragility, MCF.

Rapoport (28) has pointed out the limitations of using only the MCF to designate the osmotic resistance; and both Bolton (2) and Ecker, Hiatt, and Barr (15) have recently described the advantages of plotting the first derivative of the percentage haemolysis curve, whereby any abnormal distribution in the osmotic resistance of the erythrocytes is made evident. In the present investigation it was observed that the fragility curves frequently did not give a linear plot on probability paper. In the distribution there was often a tendency to skewness toward the lower tonicities, but never any evidence of a bimodal distribution. For comparative purposes the hypotonic fragility was recorded simply as the MCF.

## Results

### *Administration of Cholesterol*

Cholesterol was administered orally in gelatine capsules. In the first experiment it was given at the rate of 1 gm. per day for 62 days to a rabbit weighing 3.1 kgm. The plasma became very opalescent and showed a high cholesterol content, and the results of the blood analyses are summarized in Table I. A slight anaemia was produced, but the most striking observation was the parallelism between the plasma free cholesterol and the plasma AHV, as illustrated in Fig. 1. The correlation coefficient between the free cholesterol and the AHV was  $r = 0.93$ ; there was no correlation between the ester cholesterol and the AHV. In two similar experiments the rabbits survived only two weeks. In each case the plasma AHV rose as the free cholesterol increased.

TABLE I

THE EFFECT OF ORAL ADMINISTRATION OF CHOLESTEROL UPON THE AHV OF BLOOD OF A RABBIT\*

(1 gm. of cholesterol daily for 62 days)

	Cholesterol feeding				No cholesterol			
	Time in days							
	0	7	22	44	66	75	90	95
Hb, gm./100 ml.	12.9	13.2	14.0	10.8	12.1	14.3	15.0	14.9
RBC, millions/ $\mu$ l.	5.71	6.00	6.10	4.60	4.78	5.57	6.32	6.20
Haematocrit, %	39.2	39.5	37.6	31.0	38.2	41.4	45.7	43.3
MCV, $\mu^3$	69	66	62	67	80	74	72	70
Erythrocyte AHV, mgm./ml.	0.64	0.74	0.77	0.65	0.70	0.79	0.85	0.81
MCAHV, $\mu$ g/m.	0.114	0.123	0.126	0.142	0.147	0.142	0.135	0.131
Plasma AHV, mgm./ml.	2.10	3.17	5.0	9.8	7.1	4.7	3.7	3.5
Plasma cholesterol								
Free, gm./100 ml.	0.019	0.084	0.152	0.464	0.284	0.227	0.102	0.062
Ester, gm./100 ml.	0.027	0.121	0.310	0.237	0.369	0.431	0.136	—

\* In all tables: MCV = mean corpuscular volume.

MCAHV = mean corpuscular antihaemolytic value.

MCF = mean corpuscular fragility, fraction of isotonic.

These results appear to confirm the claim of Tsai and Lee (34) for the anti-haemolytic potency of the plasma free cholesterol. Van Damme (36) has found that the antihaemolytic power of the plasma seemed to parallel the concentration of cholesterol and phospholipids. However, it is noted that when the curves in Fig. 1 are extrapolated back to zero value for free cholesterol the plasma AHV is still virtually at the normal level. It is concluded that *in the rabbit* the free cholesterol normally contributes very little to the plasma AHV.

The hypercholesterolaemia following cholesterol feeding has been described by Dubach and Hill (13) and by Popják (26). The former workers noted the production of a macrocytic anaemia; and Okey (22) and Okey and Greaves (23) observed a marked anaemia in guinea pigs fed cholesterol. In the present experiments only a mild anaemia was produced. No definite changes were observed in the erythrocytes of the cholesterol-fed rabbits, apart from the anaemia and the resulting slight macrocytosis.

#### Splenectomy and Cholesterol Administration

Kennedy and Okey (18) produced a severe anaemia in guinea pigs by administering cholesterol following splenectomy. In the present experiment, two rabbits were splenectomized and cholesterol feeding (0.6 gm. per day) was commenced one month after the operation. The results obtained with one rabbit are summarized in Table II. The splenectomy produced no apparent change in the plasma cholesterol or AHV, and the only observed

change in the erythrocytes was a decrease in hypotonic fragility. Administration of the cholesterol resulted in the usual hypercholesterolaemia and a rise in the plasma AHV, as represented graphically in Fig. 1. Only a moderate anaemia followed the cholesterol feeding.

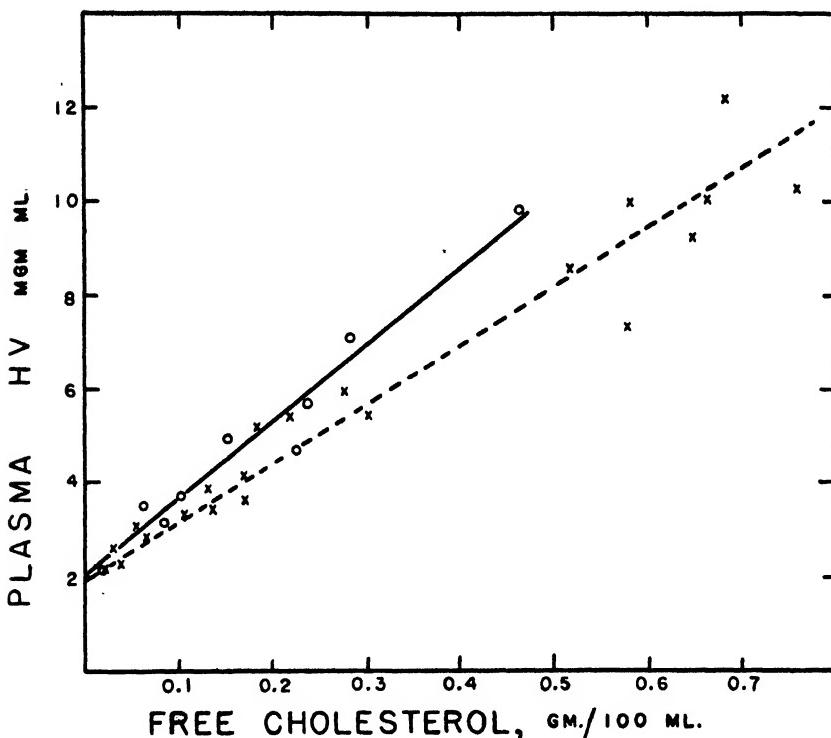


FIG. 1. The relationship between plasma free cholesterol and plasma AHV. The straight lines were determined by the method of least squares and have the equations given below.

○—○ = oral cholesterol.  $Y = 2.00 + 16.6 X$ .

×---× = splenectomy followed by cholesterol.  $Y = 1.91 + 12.8 X$ .

The second rabbit died four weeks after the commencement of cholesterol administration but the results were similar to those noted above.

#### *Effect of Repeated Bleeding*

Two rabbits were bled repeatedly by ear vein. One animal, weighing 3.0 kgm., was bled a total of 226 ml. of blood over a period of 23 days, and the results of the analyses are recorded in Table III. A marked lipaemia resulted, and again the plasma AHV paralleled the free cholesterol level. The only significant change in the erythrocytes was a macrocytosis and an elevation of the MCAHV.

The second animal, weighing 1.9 kgm., was bled a total of 188 ml. over a period of 10 days. The erythrocyte picture was similar to that observed in the first rabbit, but there were no alterations in the plasma cholesterol or AHV.

TABLE II

THE EFFECT OF SPLENECTOMY FOLLOWED BY ORAL CHOLESTEROL UPON THE AHV OF BLOOD OF A RABBIT

(0.6 gm. cholesterol daily for 16 weeks, commencing one month after splenectomy)

	Splenectomy			Cholesterol administration					No cholesterol
	Time, in weeks								
	0	1	3	5	10	16	20	24	27
Hb, gm./100 ml.	13.6	10.1	12.4	12.4	13.0	9.1	11.4	11.0	11.4
RBC, millions/ $\mu$ l.	5.54	4.64	5.41	6.30	6.16	3.60	4.72	4.88	5.84
Haematocrit, %	40.9	31.6	40.7	43.1	42.5	28.1	32.0	33.6	35.8
MCV, $\mu^3$	74	68	75	68	69	78	68	69	61
MCF	0.516	0.520	0.455	0.463	0.547	0.475	0.563	0.516	0.525
Erythrocyte AHV, mgm./ml.	0.84	0.74	0.86	0.74	0.86	0.71	0.78	0.83	0.73
MCAHV, $\mu$ g/m.	0.154	0.160	0.159	0.117	0.139	0.197	0.165	0.170	0.125
Plasma AHV, mgm./ml.	2.50	2.23	2.46	3.07	3.45	12.2	10.0	5.4	3.36
Plasma cholesterol									
Free, gm./100 ml.	0.025	0.033	0.023	0.053	0.132	0.68	0.58	0.217	0.106
Ester, gm./100 ml.	0.041	0.009	0.033	0.105	0.331	2.38	1.52	0.64	0.439

TABLE III

THE EFFECT OF REPEATED BLEEDING UPON THE AHV OF BLOOD OF A RABBIT

	Days									
	1	2	3	4	6	8	9	10	11	23
Volume bled, ml.	35	40	30	30	25	19	20	2	15	10
Hb, gm./100 ml.	12.2	10.2	7.4	6.0	5.9	6.8	7.3	7.2	6.8	13.3
RBC, millions/ $\mu$ l.	4.70	—	3.00	—	2.10	2.90	—	—	2.88	5.90
Haematocrit, %	36.0	—	22.6	—	19.8	24.4	—	—	24.0	41.0
MCV, $\mu^3$	77	—	75	—	94	84	—	—	83	70
Erythrocyte AHV, mgm./ml.	0.65	—	0.39	—	0.33	0.39	—	—	0.35	0.64
MCAHV, $\mu$ g/m.	0.138	—	0.130	—	0.155	0.135	—	—	0.120	0.109
Plasma AHV, mgm./ml.	1.83	—	1.69	—	2.34	3.07	—	—	2.19	1.87
Plasma free cholesterol, mgm./100 ml.	20	—	26	—	56	115	—	—	36	19
Plasma protein, gm./100 ml.	7.0	—	6.3	—	6.3	6.4	—	—	5.5	7.1

The hypercholesterolaemia following bleeding has already been described by the following workers: Horiuchi (17), Bloor (1), Chamberlain and Corlett (4), and Schwarz and Lichtenberg (31).

#### *Acetylphenylhydrazine Anaemia*

To investigate the blood changes in a typical haemolytic anaemia, acetylphenylhydrazine was injected intraperitoneally into four rabbits. The findings in two of the animals are represented in Fig. 2, and the results obtained with the other two animals were essentially similar.

The changes in the erythrocytes were those characteristic of a haemolytic anaemia: decrease in cell count and haemoglobin concentration, followed by an increase in the mean erythrocyte volume as immature cells were thrown

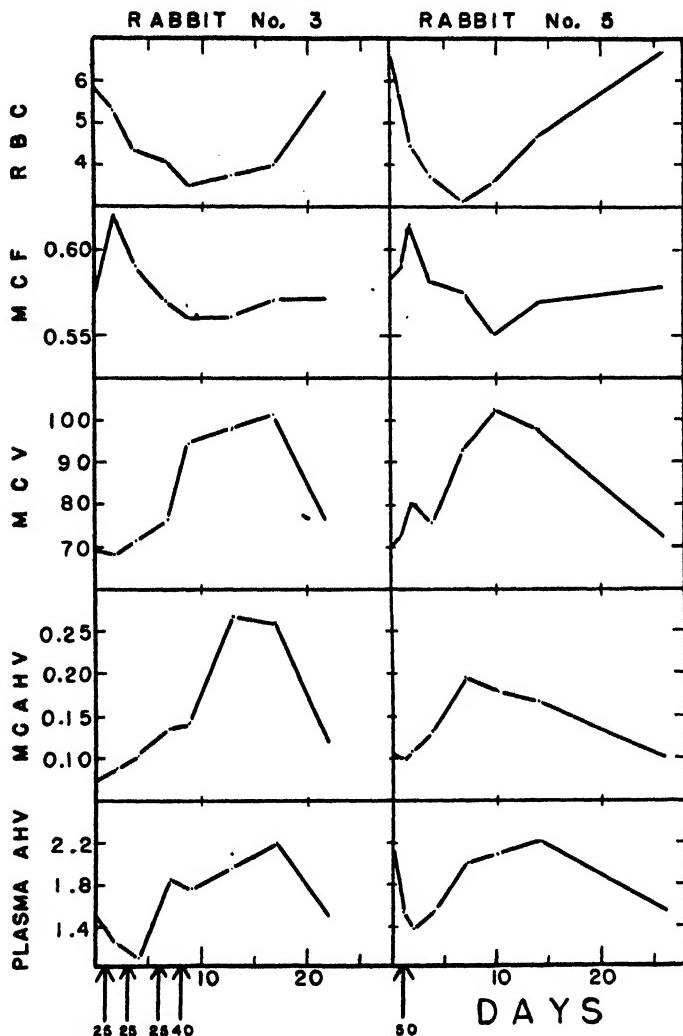


FIG. 2. The effect of acetylphenylhydrazine injection upon rabbit blood. Arrows indicate injections of acetylphenylhydrazine, in mgm.

RBC = erythrocyte count, in millions per  $\mu\text{l}$ .

MCF = mean corpuscular fragility, as a fraction of isotonic.

MCV = mean corpuscular volume, in  $\mu^3$ .

MCAHV = mean corpuscular antihaemolytic value, in  $\mu\mu\text{gm}$ .

Plasma AHV = plasma antihaemolytic value, in mgm./ml.

into the circulation. This increase in cell volume in phenylhydrazine-induced anaemia has been described by Rapoport, Guest, and Wing (29) and by Lawrason and co-workers (19).

The drug had no apparent effect upon the mean AHV (MCAHV) of the erythrocytes, other than the increase which always accompanied an increase in the MCV. In both animals, however, there was observed a slight increase in hypotonic fragility which returned to normal in a few days. Numerous Heinz bodies were observed in the erythrocytes of all animals. These are characteristic of such drug-induced anaemias and have recently been discussed by Cruz (11) and by Webster (37, 38).

The plasma AHV decreased slightly following the treatment and then returned to normal. This may possibly be accounted for by the accelerating effect of acetylphenylhydrazine upon lysolecithin haemolysis which was previously reported (7).

### Conclusions

1. The AHV of the plasma, in these experiments, was elevated by any treatment that caused an increase in plasma free cholesterol, such as oral administration of cholesterol and bleeding. It is probable that the free cholesterol neutralizes an equivalent amount of lysolecithin through the formation of a molecular complex; and experiments *in vitro* (7) have demonstrated the ability of added cholesterol to neutralize lysolecithin.

It may be noted that in Fig. 1 the molar ratios of lysolecithin to cholesterol, as represented by the slopes of the regression lines, are: 1.26 for the oral cholesterol experiment, and 0.97 for the splenectomy followed by cholesterol.

However, it must be emphasized that, although the observed *increases* in plasma AHV can be attributed to the increased free cholesterol, the free cholesterol of normal rabbit plasma apparently contributes very little to its AHV.

2. The mean antihaemolytic value (MCAHV) of the washed erythrocytes was remarkably independent of the experimental treatment, even in a haemolytic anaemia, where no decrease in the MCAHV was observed. These findings throw little light upon the mechanisms of drug anaemia, unless the observed decrease in the plasma AHV can account for increased destruction by lysins normally present. It may be noted that Dziemian (14) found an altered permeability of rabbit erythrocytes following phenylhydrazine injection, which may be related to our observation of an increase in the hypotonic fragility.

In our experiments the mean erythrocyte AHV appears to depend upon the size of the erythrocytes, and doubtless upon their composition. Dziemian (14) found that in phenylhydrazine-induced anaemia in rabbits the mean erythrocyte total lipid and free cholesterol increased as the MCV increased. It may be suggested that the mean corpuscular AHV is correlated with the cholesterol content of the erythrocytes. A rough calculation indicates that double the AHV of normal rabbit erythrocytes is of the same order of magnitude, on a molar basis, as the cholesterol content. However, changes in the erythrocyte cholesterol were not measured in our experiments.

### Acknowledgments

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# DEMONSTRATION OF AGGLUTININS FIVE HOURS AFTER INTRAPERITONEAL INJECTION OF PNEUMOCOCCUS TYPE I IN GUINEA PIGS<sup>1</sup>

By DORIS S. NUNES<sup>2</sup>

## Abstract

The inoculation of guinea pigs with pneumococci Type I intraperitoneally resulted in the development of homologous agglutinating antibodies, which were detected in the sera as early as five hours after inoculation. The early appearance of active immunity, and the attainment of a sufficient titer, would appear to govern survival to a fatal homologous re-infecting dose of the organism.

## Introduction

Type specific antipneumococcus active immunity in experimental animals is recognizable by increased resistance to homologous infection (6), as well as by the usual test-tube titrations of agglutinins, precipitins, etc. This resistance to re-infection may be present before and may persist after circulating antibodies are detectable *in vitro* (3). Circulating antibodies have been demonstrated by agglutination and other techniques in varying periods from three to four days after the injection of antigen (3, 4), and many factors have been shown to influence the amount of antibody produced and the time of its appearance.

The purpose of this paper is to report on the appearance of homologous circulating antibodies, detected by agglutination tests, in 5 and 16 hr. after injection of the antigen and before therapy was instituted (15). According to the prevailing literature, this would appear to be the earliest demonstration on record (3, 17, 9, 11).

## Materials and Methods

### *Test Organism*

A virulent strain of *Diplococcus pneumoniae* Type I was used as the infecting organism throughout these experiments.\*

### *Virulence for Guinea Pigs*

Guinea pigs are usually less susceptible to pneumococci than are mice. The pneumococcus strain S-710-TI\* showed a 100% mortality in mice in a  $10^{-7}$  dilution of an 18-hr. culture, and a 50% mortality in a  $10^{-8}$  dilution. Guinea pigs tolerated 1 ml. intraperitoneally of an 18-hr. culture without any clinical signs, and they recovered although bacteraemia was evident six hours after injection and even earlier in some of the animals. In order to ensure a fatal

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\* This strain, S-710-T I, was obtained from Dr. G. W. Rake, The Squibb Institute for Medical Research, New Brunswick, N.J.

infection in guinea pigs, phagocytosis was blocked by simultaneous intraperitoneal injection of carmine and pneumococcus culture. Carmine, given either some hours before or at the time of injection of meningococci intraperitoneally, markedly raises the apparent virulence of the culture (1, 14). Therefore, 100 mgm. carmine in 1 ml. sterile saline was injected intraperitoneally into guinea pigs followed by 1 ml. of a  $10^{-2}$  dilution of an 18-hr. culture of pneumococcus Type I containing approximately 12.5 million viable organisms per ml. which produced a 50% mortality rate in guinea pigs. Viability counts were done by "the drop method" described by Reed and Reed (18).

### *Animals*

Groups of guinea pigs, all weighing between 500-600 gm. were chosen. These were kept under observation for at least a week prior to infection, and a preliminary bleeding taken in order to determine that no specific circulating antibodies for the test organism existed.

### *Agglutination Tests*

Halving serial dilutions of each serum in 0.2 ml. volume in 0.85% saline ranging from a 1 in 2 dilution to a 1 in 2048 dilution were made in clean dry Kahn tubes; the control containing 0.2 ml. saline only. An equal volume of standard antigen was added to all tubes, which were placed in a water bath at 37° C. for two hours and then in the refrigerator overnight at 4° C. The tubes were read for agglutination against a viewing-box having a black background with oblique light.

### *Preparation of the Antigen*

About 100 ml. of "I.S."\* broth was inoculated heavily with lyophilized S-710-TI pneumococci cultures and incubated at 37° C. for 18 hr. The cultures were killed by heating for one hour at 60° C. and centrifuged. The organisms were washed four times with 0.85% saline, and finally diluted with saline to a concentration of No. 2 McFarland nephelometer and stored in the refrigerator at 4° C. The antigen was finally tested for Gram-positiveness, capsule swelling, and for sterility by aerobic and anaerobic cultures. In addition, no antigen more than a week old was ever used.

### *Variation in Consecutive Antigen Preparations*

Several lots of antigen had to be prepared during a series of experiments, and many showed variation in agglutinability when prepared by the same or different procedures. Variation in agglutinability could not be directly related to size of tubes, temperature, pH, medium, time, shaking on addition of antigen, and virulence of the organisms, all of which were checked repeatedly. Instead of compensating for this, by assigning to each preparation an agglutinability factor, which might be difficult in weak sera, a standard immune serum was used, to select antigens of suitably sensitive agglutinability (See Fig. 1).

\* Special culture media used in the Department of Bacteriology, McGill University.

### LEGEND TO FIGURES

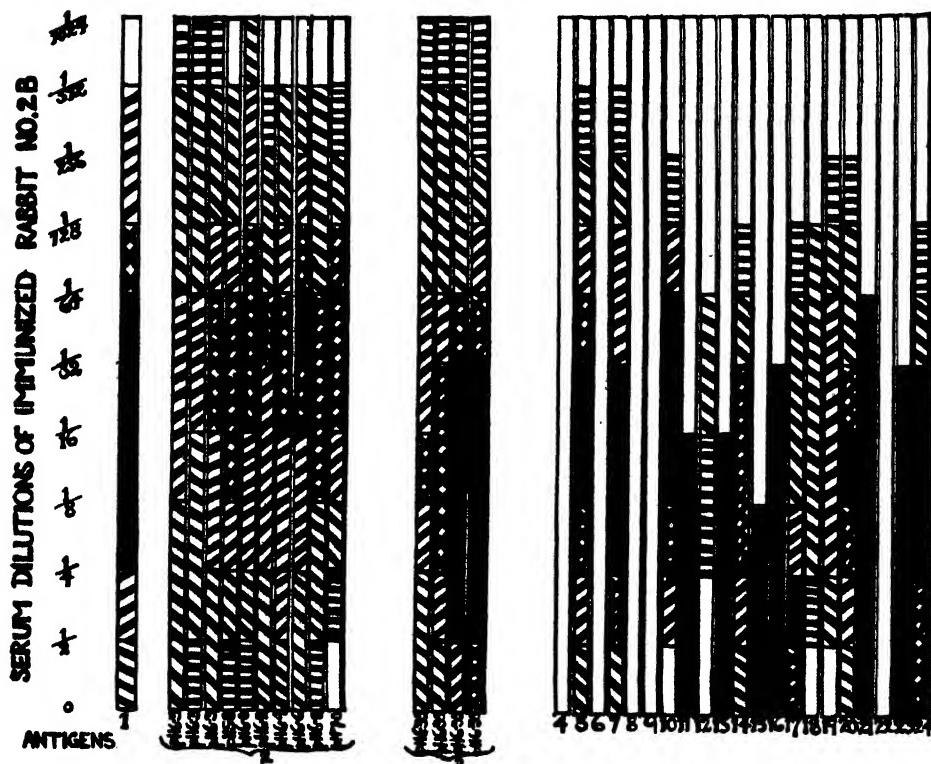
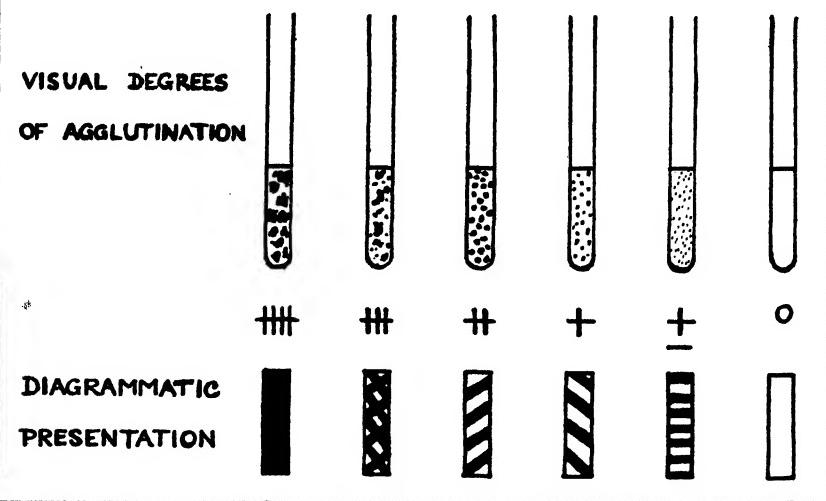


FIG. 1. Variation of agglutinability of pneumococci Type I antigens, using the same immunized Type I pneumococcal rabbit serum.

## Experimental

### AGGLUTINATING ANTIBODIES FIVE HOURS AFTER INTRAPERITONEAL INJECTION OF PNEUMOCOCCUS TYPE I

#### Procedure

Selected guinea pigs (No. S1-S18 inclusive) were bled by cardiac puncture five hours after the standardized intraperitoneal injection of carmine and pneumococcus Type I culture. In five hours, the animals all showed clinical signs of infection as seen by ruffled fur, lethargy, and apathy to stimulation. The infection was then controlled by sulphamethazine immediately after this bleeding and for the next four to five days.

Another group of guinea pigs (No. P1, P3-P26 inclusive) were similarly injected intraperitoneally, then bled in five hours. Penicillin was the therapeutic agent used in this group.

Subsequent bleedings were taken in 5, 10, and 20 days after infection in both groups of animals, and in addition, cutaneous tests to Type I capsular polysaccharide,\* and homologous re-infection with the original infecting dose were made on the 20th day.

Cutaneous tests were made by the intracutaneous injection of 0.1 ml. containing 0.0004 mgm. of Type I capsular polysaccharide on the shaved abdominal wall. A positive reaction was taken as an area of erythema surrounding the site of injection, usually appearing within a half hour, with absence of any reaction in the control which consisted of saline only.

A third group of guinea pigs (No. I1-I5 inclusive) were injected in the same way, then immediately afterwards given their first dose of sulphamethazine. These animals were bled in five hours, again in 16 hr., in five days, 10 and 20 days. These were similarly subjected to cutaneous tests with Type I polysaccharide and re-infection on the 20th day.

#### Results

In the S series, seven out of the 18 sera showed agglutinating antibodies varying from a 1 in 32 dilution to a 1 in 256 dilution. These were not very high in amount, as + agglutination was the highest recorded, and all except one serum showed prezung. Eleven of the sera were negative in all dilutions.

In the P series, there were demonstrable agglutinating antibodies in five out of 25 sera, varying from a 1 in 4 dilution to a 1 in 64 dilution, with ++ agglutination the highest recording, and only two sera showed prezung. Twenty sera were negative in all dilutions (Fig. 2).

In the I series, none of the sera exhibited agglutination.

In addition, it was noted that the antibody titer achieved by the 20th day in these series of animals appeared to have some definite bearing on the response of the animals to re-infection (Table I) viz.: guinea pigs S3, S6,

\* Obtained from the Squibb Institute for Medical Research, New Brunswick, N.J.

TABLE I

## COMPARISON OF CUTANEOUS TESTS AND RE-INFECTION TO THE ANTIBODY TITER ON THE 20TH DAY

Guinea pig No.	Antibody titer (five hours)	Antibody titer (20th day)	Cutaneous test to Type I capsular polysaccharide (20th day)	Survival to re-infection (20th day)
S 3	1 : 32	(Died)	O	O
S 6	1 : 128	(Died)	O	O
S 8	1 : 128	1 : 32	+	+
S 15	1 : 128	1 : 1024	+	+
S 16	1 : 256	1 : 512	+	+
P 17	1 : 32	1 : 512	+	+
S 18	1 : 32	1 : 512	+	+
P 8	1 : 8	(Died)	O	O
P 9	1 : 4	(Died)	O	O
P 11	1 : 4	(Died)	O	O
P 18	1 : 64	1 : 512	+	+
P 20	1 : 16	1 : 512	+	+
I 1	0	1 : 64	+	+
I 2	0	1 : 128	+	+
I 3	0	(Died)	O	O
I 4	0	1 : 256	+	+
I 5	0	1 : 256	+	+

P8, P9, P11, I3. These six animals demonstrated titers varying from 0 to 1 in 128 dilution in five hours, however, did not survive until the 20th day. Those that did survive demonstrated titers of 1 in 64 dilution or higher by the 20th day, with the exception of guinea pig S8, and were able to resist re-infection.

#### AGGLUTINATING ANTIBODIES 16 HR. AFTER INTRAPERITONEAL INJECTION OF PNEUMOCOCCUS TYPE I

##### *Procedure*

Guinea pigs (Nos. S'1-S'20 inclusive) were bled 16 hr. after the standardized intraperitoneal injection of carmine and pneumococcus Type I culture. Clinical signs of infection were more marked than in five hours, as may be expected. Sulphamethazine was then administered as in the S series.

Guinea pigs (Nos. P'2-P'15 inclusive) were similarly injected intraperitoneally, then bled in 16 hr. after which the first dose of penicillin was given as in the P series.

Both these groups, S' and P', were similarly bled in 5, 10, and 20 days after injection, and tested cutaneously with Type I capsular polysaccharide and homologous re-infection on the 20th day.

A third group of guinea pigs (Nos. I1-I5 inclusive) were injected in the same way, then immediately afterwards given their first dose of sulphamethazine. These animals were bled in five hours, again in 16 hr., in five

days, 10 and 20 days. These were similarly subjected to cutaneous tests with Type I polysaccharide and re-infection on the 20th day.

### Results

In the S' series, four out of 20 animals showed agglutinating antibodies varying from a 1 in 8 dilution to a 1 in 64 dilution with pre zoning in one serum only, and ++ agglutination was the highest reading. Sixteen sera were negative in all dilutions.

In the P' series, agglutinating antibodies could be demonstrated in 6 out of 14 sera. The titers in this group were the highest obtained, varying from a 1 in 8 dilution to a 1 in 2048 dilution, with ++ agglutination the highest recording, and three out of six sera exhibiting pre zoning. Eight sera were negative in all dilutions (See Fig. 2).

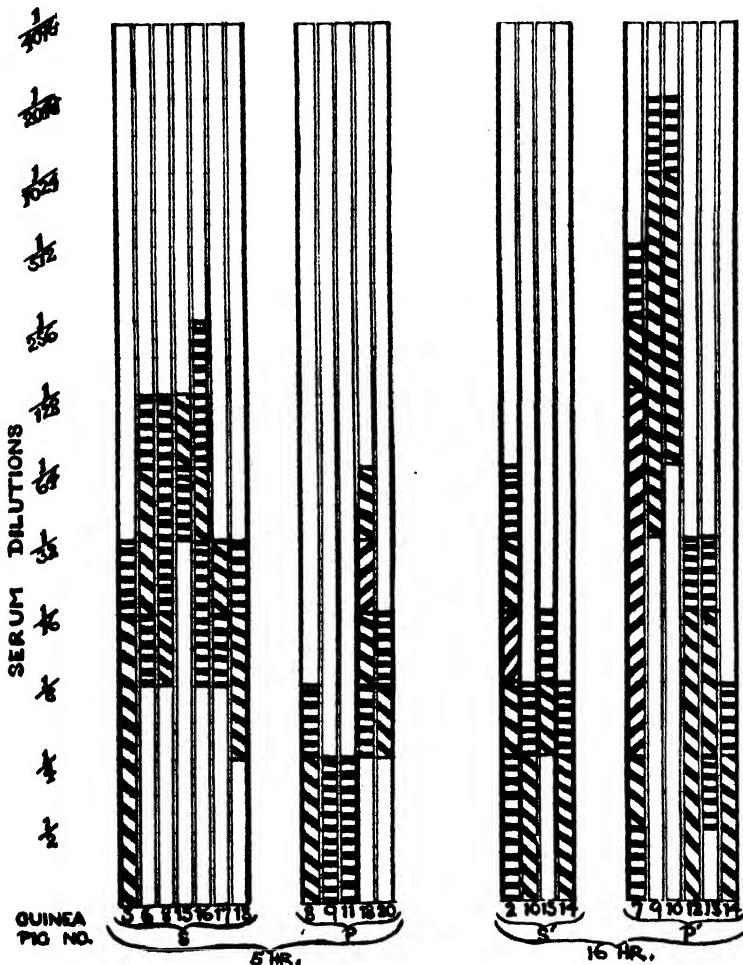


FIG. 2. Agglutination titers in five hours and 16 hr. after inoculation of Type I pneumococci intraperitoneally in guinea pigs.

In the I series, none of the sera exhibited agglutination.

As in the previous series, the titer at time of re-infection bore a definite relationship to survival and therefore some prediction of the outcome could be made (See Table II).

TABLE II

COMPARISON OF CUTANEOUS TESTS AND RE-INFECTIOON TO THE ANTIBODY TITER ON THE 20TH DAY

Guinea pig No.	Antibody titer (16 hr.)	Antibody titer (20th day)	Cutaneous Test to Type I capsular polysaccharide (20th day)	Survival to re-infection (20th day)
S' 2	1 : 64	(Died)	0	0
S' 10	1 : 8	1 : 1024	+	+
S' 13	1 : 16	1 : 1024	+	+
S' 14	1 : 8	1 : 128	+	+
P' 7	1 : 512	1 : 128	+	+
P' 9	1 : 2048	1 : 128	+	+
P' 10	1 : 2048	1 : 64	+	+
P' 12	1 : 32	1 : 8	+	0
P' 13	1 : 32	(Died)	0	0
P' 14	1 : 8	1 : 128	+	+
I 1	0	0 : 64	+	+
I 2	0	1 : 128	+	+
I 3	0	(Died)	0	0
I 4	0	1 : 256	+	+
I 5	0	1 : 256	+	+

In these groups, only guinea pig P'12 had a titer of 1 in 8 dilution by the 20th day, and failed to survive re-infection; those surviving demonstrated a titer of 1 in 64 dilution or higher by the 20th day.

In addition to the titer finally achieved by the 20th day, there appeared to be a further relationship between the *early* formation of antibodies and a greater resistance to re-infection (See Table III).

TABLE III

EARLY APPEARANCE OF ANTIBODIES AND RESISTANCE TO RE-INFECTIOON IN GUINEA PIGS

	5-Hr. group		16-Hr. group	
	Antibody present	No antibody present	Antibody present	No antibody present
Total No. of guinea pigs	12	36	.	.
No. guinea pigs dying before 20th day	5	20	2	16
No. guinea pigs surviving to 20th day	7	16	8	13
No. guinea pigs resistant to re-infection on 20th day	7	11	7	11

### Discussion

The higher survival rate of the guinea pigs which formed antibodies in less than 16 hr. indicated a greater resistance to re-infection than those animals which were tardy in producing antibodies. In the latter group, only 29 out of 65 survived until the 20th day, and of these 22 only resisted re-infection. This was in contrast to those early antibody producers, of which 15 out of 22 survived until the 20th day, and then only one succumbed to re-infection.

In guinea pigs (11-5 inclusive) which received sulphonamides immediately after infection, the first appearance of antibodies was late (10th day), and these animals appeared resistant to re-infection on the 20th day. In view of the small group of animals used here, it is difficult to draw any conclusions as to their real or apparent resistance.

The toxicity of penicillin for guinea pigs as a species (8, 16) was a definite handicap, and produced per se a higher mortality in the penicillin treated animals. However, this factor was present for both the early and late antibody formers in the P and P' series. It was also noted that five out of 39 of the guinea pigs treated with penicillin demonstrated grossly fatty sera, in which estimations for fatty acid gave results ranging from 30 to 285 milk equivalents (13).

It would therefore appear that the early evidence of an immune response, as demonstrated by circulating antibodies, would predict a lasting and effective state of immunity, and resistance to re-infection. This was shown in the lower mortality rate of this group when re-infected with the original re-infecting dose, as compared to those in which antibodies were later in appearing. In addition to this, the titer at time of re-infection was an important criterion even in those animals which produced antibodies within 16 hr., and, for the standard antigen used, a maximum titer of a 1 in 8 dilution was insufficient to ensure protection. A safe minimum was found to be agglutination in a 1 in 64 dilution.

No definite explanation for the remarkably early appearance of circulating antibodies can be offered. The reticuloendothelial system (9, 10) and lymphocytes (2, 7) have been credited with the production of antibodies in the body. Hoder (9) has shown that damage to the reticuloendothelial system resulted in a decrease in antibody production, and stimulation in an increase. Whereas carmine was used effectively in raising the apparent virulence of the organism, it may also play some role in maintaining antibody formation. Several unrelated adjuvants have been used to sustain antibody formation (5), by probably protecting the antigen against destruction and elimination. However to attribute carmine with the property of increasing the rate of antibody production, further investigation would be necessary. Carmine does not appear to affect the apparent health of mice. It is first "agglutinated" into masses, phagocytosed, and eventually removed by the peritoneum and omentum. The response of the animals to such an early production of antibodies appeared almost in the nature of an allergic or hypersensitive reaction.

Preliminary bleedings, however, ruled out any possibility of previous sensitization to the test organism, and no adverse clinical after-effects were noted.

Löfström (12) has reported a nonspecific capsular swelling substance of pneumococcus formed in the early stages of bacterial disease, and associated with the  $\alpha$  and  $\beta$  globulin fractions of serum proteins. This substance is produced after the subcutaneous injection of pneumococci Types I and III in rabbits. It appears within 24 hr., attaining a maximum in two days, disappears in a week, and agglutinates pneumococci Types XVI, XXVII, and XXVIII. However, this substance has not been demonstrated in horses or guinea pigs.

Assuming that favorable conditions existed in the choice of the antigen, and the route chosen permitted a large absorptive area, the agglutinating titers obtained in 5 and 16 hr. were enormous. Furthermore, the variability of the antigen was controlled, so no untoward sensitivity can be blamed.

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